Universidade de Aveiro Departamento de Química
2012

Sónia Andreia Oliveira Santos

Compostos fenólicos a partir de subprodutos da indústria florestal

Phenolic compounds from forest industrial by-products

Universidade de Aveiro
2012

Departamento de Química

Sónia Andreia Oliveira Santos

Compostos fenólicos a partir de subprodutos da indústria florestal
Phenolic compounds from forest industrial by-products

Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Química, realizada sob a orientação científica do Doutor Carlos Pascoal Neto, Professor catedrático do Departamento de Química da Universidade de Aveiro e do Doutor Armando Jorge Domingues Silvestre, Professor associado com agregação do Departamento de Química da Universidade de Aveiro

Apoio financeiro do projeto AFORE (CP-IP 228589-2) no âmbito do VII Quadro Comunitário de Apoio.

Apoio financeiro do projeto WaCheUp (NMP-TI-3 – STRP 013896) no âmbito do VI Quadro Comunitário de Apoio.

Apoio financeiro da FCT e do FSE (SFRH / BD / 42021 / 2007), no âmbito Humano (POPH) do QREN.

À minha mãe.

o júri

presidente

Doutor Carlos Alberto Diogo Soares Borrego
professor catedrático da Universidade de Aveiro

Doutor Carlos Pascoal Neto
professor catedrático da Universidade de Aveiro

Doutor Nuno Filipe da Cruz Batista Mateus
professor associado com agregação da Faculdade de Ciências da Universidade do Porto

Doutor Armando Jorge Domingues Silvestre
professor associado com agregação da Universidade de Aveiro

Doutora Maria do Rosário Reis Marques Domingues
professora auxiliar da Universidade de Aveiro

Doutora Paula Cristina de Oliveira Rodrigues Pinto
investigadora auxiliar da Faculdade de Engenharia da Universidade do Porto

agradecimentos

Os meus agradecimentos vão, em particular, para o Professor Doutor Armando J. D. Silvestre e Professor Doutor Carlos Pascoal Neto, pela excelente orientação científica, apoio incondicional e, principalmente, por toda a confiança que depositaram em mim.

À Professora Doutora Maria Rosário Domingues, agradeço todo o apoio e entusiasmo que me deu ao longo destes anos e de forma incansável. Agradeço também a preciosa ajuda na espectrometria de massa.

Gostaria de agradecer ao Professor Doutor Carlos Silva por todo o apoio na extração supercrítica e pela confiança que depositou em mim.

À Doutora Paula Pinto faço um especial agradecimento por tudo o que me transmitiu no início deste trabalho, por toda a sua amizade e, acima de tudo, pela sua sempre boa disposição.

À Doutora Carmen Freire agradeço todo o apoio, os conselhos e incentivos.

Gostaria de agradecer ao Doutor Juan Jose Villaverde por todo o apoio, amizade e incentivo que me facultou, sempre de forma incansável.

Ao grupo de espectrometria de massa do Departamento de Química da Universidade de Aveiro gostaria de fazer um especial agradecimento por toda a sua disponibilidade, em particular, ao Professor Doutor Pedro Domingues, pela preciosa ajuda com o HPLC.

Gostaria de agradecer a todos os meus colegas de trabalho, aos dos “nossos” laboratórios e aos dos laboratórios ao lado, aos que ainda lá trabalham e aos que já foram. Todos tornaram todos estes anos tempos excecionais.

Ao Ricardinho, à Carla, à Andreinha, ao Rui, à Lili, à Mónica, à Bi, à Gi e à Belinda, faço um especial agradecimento, por terem tornado todo este percurso muito mais fácil.

A todos os que me ouvirem dizer infinitas vezes “Good morning, my name is Sónia…”, agradeço a enorme paciência. À Nor, ao Tiago, ao Fernando, à Vera, ao Ricardo, ao Nelson, à Vânia e à Joana, que de forma incansável sempre me apoiaram e animaram, faço um especial agradecimento. Agradeço, acima de tudo, por compreenderem tantas ausências. Ao João Paulo, agradeço o facto de me ter transmitido ao longo destes anos que com força de vontade nenhum obstáculo é suficientemente grande.

Por último, mas não menos importante, agradeço à minha família, em particular aos meus pais e irmãos por todo o apoio e principalmente por compreenderem quando eu não pude estar. Agradeço especialmente ao Tiago, por inúmeras razões, entre as quais todo o incansável apoio, compreensão e, claro, muita paciência.

palavras-chave

Compostos fenólicos, *Quercus suber* L., cortiça, *Eucalyptus globulus* Labill., *Eucalyptus grandis*, *Eucalyptus urograndis (Eucalyptus grandis x Eucalyptus urophylla)*, *Eucalyptus maidenii*, eucalipto, HPLC-MS, atividade antioxidante, extração supercrítica.

resumo

Em Portugal, as indústrias corticeira e de pasta de papel constituem um importante sector económico, contudo, gerando elevadas quantidades de subprodutos. Estes subprodutos poderiam ser explorados em aplicações de alto valor acrescentado, como fonte de compostos fenólicos, por exemplo, em vez de serem apenas queimados para produção de energia. Estes compostos são conhecidos pelas suas inúmeras propriedades, entre as quais, antioxidante, anti-inflamatória e anti-trombótica.

Neste estudo as frações fenólicas da maior parte dos subprodutos gerados nas indústrias corticeira e de pasta de papel foram caracterizados em detalhe, com vista à sua valorização. A fração fenólica das cascas de *Eucalyptus globulus*, *E. grandis*, *E. urograndis* e *E. maidenii*, bem como da cortiça de *Quercus suber* e resíduos provenientes da sua exploração, nomeadamente, o pó de cortiça e os condensados negros, foi obtida por processos convencionais de extração sólido-líquido.

No caso da casca de *E. globulus*, foi ainda avaliado o potencial de metodologias “verdes” no processo de extração de compostos fenólicos, usando extração com CO_2 supercrítico. Esta técnica foi otimizada com recurso a metodologias de superfície de resposta.

Na identificação e quantificação dos compostos fenólicos foi usada cromatografia líquida de alta resolução aliada a técnicas de espectrometria de massa. O teor de fenólicos totais foi ainda determinado pelo método de Folin-Ciocalteu, essencialmente para efeitos comparativos. A caracterização da fração fenólica de cada extrato foi ainda complementada com a análise da atividade antioxidante, usando o radical 2,2-difenil-1-picrilhidrazilo (DPPH).

Foram identificados trinta compostos fenólicos na casca de *E. globulus*, 17 deles referenciados pela primeira vez como seus constituintes, nomeadamente os ácidos quínico, di-hidroxifenilacétic, cafeico e metil-elágico, bis-hexa-hidroxidifenoil(HHDP)-glucose, galoil- bis-HHDP-glucose, galoil-HHDP-glucose, isoramnetina—hexosídeo, quercetina-hexosídeo, ácido metil-elágico-pentosídeo, miricetina-ramnosídeo, isoramnetina-ramnosídeo, mearnsetina, floridzina, mearnsetina-hexosídeo, luteolina e uma proantocianidina B.

Neste trabalho, foi estudada pela primeira vez a composição fenólica das cascas de *E. grandis*, *E. urograndis* e *E. maidenii*. (cont.)

resumo (cont.)

Treze, doze e vinte e quatro compostos fenólicos foram identificados nas cascas de *E. grandis*, *E. urograndis* e *E. maidenii*, respetivamente. Entre estes compostos encontram-se os ácidos quínico, gálico, metilgálico, protocatequínico, clorogénico e elágico, catequina, galoil-bis-HHDP-glucose, digaloilglucose, epicatequina, quercetina-glucoronídeo, di-hidroxi-isopropilcromona-hexosídeo, isoramnetina-hexosídeo, ácido elágico-ramnosídeo, taxifolina, quercetina-hexosídeo, di-hidroxi-(metilpropil)isopropilcromona-hexosídeo, ácido metil-elágico-pentosídeo, miricetina-ramnosídeo, isoramnetina-ramnosídeo, aromadendrina-ramnosídeo, mearnsetina, mearnsetina-hexosídeo, eriodictiol, quercetina, isoramnetina e naringenina. A análise da fração fenólica da cortiça permitiu identificar vinte e dois compostos fenólicos, dez deles referenciados pela primeira vez como seus constituintes, nomeadamente, os ácidos quínico, salicílico, *p*-hidroxifenil-lático e metilgálico, ácido carboxílico da brevifolina, eriodictiol, naringenina, um éster isoprenílico do ácido cafeico, isoramnetina-ramnosídeo e isoramnetina. No pó de cortiça industrial foram identificados dezasseis compostos fenólicos, nomeadamente os ácidos quínico, gálico, protocatequínico, cafeico, ferúlico, elágico e metilgálico, esculetina, ácido carboxílico da brevifolina, coniferaldeído, um éster isoprenílico do ácido cafeico, uma dilactona do ácido valoneico, ácido elágico-pentosídeo, ácido elágico-ramnosídeo, isoramnetina-ramnosídeo e isoramnetina. Destes, apenas o ácido elágico foi previamente referenciado como componente do pó de cortiça. Do mesmo modo, treze compostos fenólicos foram identificados no condensado negro, doze deles referenciados pela primeira vez como seus constituintes. São eles os ácidos quínico, gálico, *p*-hidroxifenil-láctico, protocatequínico, *p*-coumarico, cafeico e elágico, vanilina, esculetina, coniferaldeído, um éster isoprenílico do ácido cafeico e o eriodictiol.

A extração supercrítica de compostos fenólicos da casca de eucalipto permitiu não só verificar os parâmetros que afetam a qualidade e quantidade finais dos extratos, como também obter os valores ótimos para estes parâmetros. Esta extração mostrou ainda ser bastante seletiva para determinados grupos de compostos fenólicos, como as flavanonas eriodictiol e naringenina e para o flavonol *O*-metilado isoramnetina.

Este é também o primeiro estudo envolvendo a determinação da atividade antioxidante de extratos da cortiça e dos resíduos da sua exploração, bem como da casca de *E. grandis*, *E. urograndis* e *E. maidenii*.

A vasta gama de compostos fenólicos identificados em cada extrato analisado, assim como as prominentes atividades antioxidantes, todas na mesma gama de valores do bem conhecido antioxidante comercial, ácido ascórbico, são claramente um grande contributo para a valorização destes subprodutos industriais.

keywords

Phenolic compounds, *Quercus suber* L., cork, *Eucalyptus globulus* Labill., *Eucalyptus grandis*, *Eucalyptus urograndis* (*Eucalyptus grandis x Eucalyptus urophylla)*, *Eucalyptus maidenii*, HPLC-MS, antioxidant activity, supercritical extraction.

abstract

In Portugal, the cork and the pulp and paper industries are important economic sectors, however, generating substantial amounts of by-products. These by-products could be exploited in added value applications, rather than being simply burned for energy production, as, for example, as a source of the valuable phenolic compounds. These compounds are known by their innumerous properties, as antioxidant, anti-inflammatory or even anti-thrombotic.

In this study, the phenolic fractions of the most abundant cork and pulp industrial residues were characterised in detail, aiming at up-grading them. The phenolic fraction of the barks of *Eucalyptus globulus*, *E. grandis*, *E. urograndis* and *E. maidenii* as well as the cork from *Quercus suber* and the residues of its exploitation, namely, cork powder and black condensates, were obtained by conventional solid-liquid extractions.

In the case of *E. globulus* bark, the potential application of green methodologies in the extraction of phenolic compounds was also evaluated, by using supercritical CO_2 extraction. This approach was optimized by using surface response methodology.

High-performance liquid chromatography coupled with mass spectrometry techniques were used in the identification and quantification of phenolic compounds. The total phenolic content was also accessed by the Folin-Ciocalteu method, mainly for comparative purposes. The characterization of the phenolic fraction of each extract was also complemented with antioxidant activity measurements, by using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging.

Thirty phenolic compounds were identified as constituents of *E. globulus* bark, 17 of them referenced for the first time, namely, quinic, dihydroxyphenylacetic, and caffeic acids, bis-hexahydroxydiphenoyl(HHDP)-glucose, galloyl-bis-HHDP-glucose, galloyl-HHDP-glucose, isorhamentin-hexoside, quercetin-hexoside, methyl-ellagic acid, methyl-ellagic acid (EA)-pentoside, myricetin-rhamnoside, isorhamnetin-rhamnoside, mearnsetin, phloridzin, mearnsetin-hexoside, luteolin and a proanthocyanidin B-type dimer.

The phenolic composition of *E. grandis*, *E. urograndis* and *E. maidenii* bark was studied in this work for the first time. Thirteen, twelve and twenty four phenolic compounds were identified in *E. grandis*, *E. urograndis* and *E. maidenii* bark extracts, respectively. These compounds include quinic gallic, protocatechuic, chlorogenic and ellagic acids, methyl gallate, catechin, galloyl-bis-HHDP-glucose, digalloylglucose, epicatechin, quercetin-glucuronide, dihydroxy-isopropylchromone-hexoside, isorhamnetin-hexoside, ellagic acid-rhamnoside, taxifolin, quercetin-hexoside, dihydroxy-(methylpropyl)isopropylchromone-hexoside, methyl-ellagic acid-pentoside, myricetin-rhamnoside, isorhamnetin-rhamnoside, aromadendrin-rhamnoside, mearnsetin, mearnsetin-hexoside, eriodictyol, quercetin, isorhamnetin and naringenin. (cont.)

abstract (cont.)

The analysis of the phenolic fraction of cork allowed to identify twenty two phenolic compounds, ten of them reported for the first times as its constituents, namely, quinic, salicylic and *p*-hydroxyphenyl-lactic acids, eriodictyol, naringenin, methyl gallate, brevifolin carboxylic acid, caffeic acid isoprenyl ester, isorhamnetin-rhamnoside and isorhamnetin. It were identified sixteen phenolic compounds in industrial cork powder, namely, quinic, gallic, protocatechuic, caffeic, ferulic and ellagic acids and methyl gallate, esculetin, brevifolin carboxylic acid, coniferaldehyde, caffeic acid isoprenyl ester, valoneic acid dilactone, ellagic acid-pentoside, ellagic acid-rhamnoside, isorhamnetin-rhamnoside and isorhamnetin. From these, only ellagic acid was previously reported as constituent of cork powder. Likewise, thirteen phenolic compounds were identified on black condensate, twelve of them for the first time, namely quinic, gallic, *p*-hydroxyphenyl-lactic, protocatechuic, *p*-coumaric, caffeic and ellagic acids and vanillin, esculetin, coniferaldehyde, caffeic acid isoprenyl ester and eriodictyol.

The supercritical extraction of phenolic compounds from *E. globulus* bark allowed to verify the parameters affecting the qualitatively and quantitatively the final extracts. The optimal conditions of those parameters were obtained. This technique showed to be selective to restrict classes of compounds, such as flavanones and *O*-methylated flavonols.

This was also the first study involving the evaluation of the antioxidant activity of the phenolic extracts of *E. grandis*, *E. urograndis* and *E. maidenii* bark as well as of cork and the residues of their exploitation.

The vast range of phenolic compounds identified in each vegetal source studied, as well as its outstanding antioxidant activities, all in the same range of the well known commercial antioxidant ascorbic acid, are, clearly, a contribute to the up-grading of these industrial by-products.

Table of contents

Abbreviations/Acronyms

λmax – wavelength for which a compound has a maximum ultraviolet absorbance

AA – antioxidant activity

AAE – ascorbic acid equivalents

AAPH – 2,2‘-azobis(2-amidinopropane) dihydrochloride

ABTS – 2,2‘-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)

AOP –antioxidant potential

APCI – atmospheric pressure chemical ionisation

ASE – accelerated solvent extraction

BC – black condensate

BHA – butylated hydroxyanisole

BHT – butylated hydroxytoluene

BHQ – *tert*-butylhydroquinone

CE – capillary electrophoresis

CUPRAC – cupric reducing antioxidant capacity

CZE – capillary zone electrophoresis

DAD – diode array detector

DPPH – 2,2-diphenyl-1-picrylhydrazyl

DNA – deoxyribonucleic acid

EA – ellagic acid

ET – electron transfer

EtOAc – ethyl acetate

EtOH – ethanol

ESI – electrospray ionisation

EY – extraction yield

FAB – fast atom bombardment

FID – flame ionization detector

FL – fluorescence

FRAP – ferric reducing antioxidant power

FT-ICR – fourier transform ion cyclotron resonance

GAE – gallic acid equivalents

GC – gas-chromatography

HAT – hydrogen atom transfer

HHDP – hexahydroxydiphenoyl

HPCE – high-performance capillary electrophoresis

HPLC – high-performance liquid chromatography

ICP – industrial cork powder

LCF – lignocellulosic feedstock

LOD – limit of detection

LOQ – limit of quantification

m/*z* – mass-to-charge ratio

MAE – microwave-assisted extraction

MALDI – matrix assisted laser desortion

MeOH – methanol

MS – mass spectrometry

MS/MS – tandem mass spectrometry

MS^n – multistage mass spectrometry

NC – natural cork

NMR – nuclear magnetic ressonance

NP – normal phase

ORAC – oxygen radical absorbance capacity

PC-HPLC – phenolic content, quantified by HPLC

PD – plasma desorption

PLE – pressurized liquid extraction

QqQ – triple quadrupole mass analyser

RP – reversed-phase

R-PE – R-phycoerythrin

SEC – size exclusion chromatography

SFC – supercritical fluid chromatography

SFE – supercritical fluid extraction

SLE – solid-liquid extraction

SPE – solid phase extraction

SPME – solid phase microextraction

TBA – thiobarbituric acid

TEAC – trolox equivalents antioxidant capacity

TLC – thin layer chromatography

TPC – total phenolic content, quantified by Folin-Ciocalteu method

TPTZ – 2,4,6-tripyridyl-s-triazine

TRAP – total peroxyl radical-trapping antioxidant parameter

TSP – thermospray ionisation

ToF – time of flight

TPTZ – 2,4,6-tri(2-pyridyl)-s-triazine

UHPLC – ultra high-performance liquid chromatography

US – ultrasounds

USAE – ultrasound-assisted extraction

UV – ultraviolet

UV-Vis – ultraviolet-visible

Chapter 1

Introduction

1.1 Biorefinery context

Over the last years there is a large increase on the search of new solutions to the inevitable depletion of fossil resources, coupled with the growing interest on environmental concerns. These have been lead to an emerging research trend in the search of bio-based products, within the biorefinery concept [1-6].

1.1.1 Biorefinery concept

“A biorefinery is a facility that integrates biomass conversion processes and equipment to produce fuels, power, and chemicals from biomass”- definition of biorefinery by the National Renewable Energy Laboratory [7]. There are many definitions of this term, however, all of them converging to the same goal: transform different biomass feedstock into useful products by using diverse technologies and processes [1, 4, 8]. Despite, in the last decade, this term has been widely used, this concept is not recent. The novelty is to use biomass (products from agricultural commodities) to produce a wide range of end-products (fuels, power, materials and chemicals), by using complex process as occurs in a petroleum refinery [8]. Ideally, a biorefinery should combine together chemical/biochemical and thermal conversion processes to convert biomass into a wide variety of products as well power for its own use and ideally for exportation to the network [8] Several biomass sources have been focused, such as wood and forest materials (lignocellulosics), agriculture crops and wastes, agro-food industries wastes or aquatic plants [1, 5].

1.1.2 Biorefinery classification

Biorefineries have been classified into phase I, II and III biorefineries, according to the feedstock’s used [4, 8]. A phase I biorefinery has a fixed process capability, and uses only one feedstock as raw material. A dry-milling ethanol plant is an example of this type of biorefineries, which are already considered economically viable. A biorefinery plant, using also grain as feedstock, but with the capability to produce a range of end-products, as the wet-milling technology, is classified as a phase II biorefinery. A phase III biorefinery, the most advanced and flexible, uses different types of feedstock’s and technologies and has the capability to produce various products, as chemicals and/or fuels [4, 8]. Currently three phase III biorefinery systems, classified according to their processed raw materials, have been object of research and development [1, 4]:

"Lignocellulosic feedstock biorefinery (LCF) – uses fibrous plants materials, such as wood, straw or corn stover, which are firstly fractionated into their main fractions (hemicelluloses, cellulose, lignin and extractives) and further converted into chemicals or fuels/energy;

Whole-crop biorefinery – uses cereals, as rye or maize as raw material. This type of biorefinery uses the entire plant to produce useful products, materials and energy. The first step involves the separation of the seed from the straw. The seeds may be processed to yield starch or other wide variety of products, while straw is used as raw material in a LCF biorefinery; and

Green biorefinery – uses natural-wet feedstock's, as green plants and crops in a multi-product system, which handle its fractions and products, according to the physiology of the plant material.

The LCF biorefinery has shown to be the most promising as it has the potential to accommodate a wide range of raw materials at a competitive price (straw, reed, grass, wood, paper-waste, etc.) that can yield conversion products in both the traditional petrochemical-dominated and the future bio-based product markets [2]. Forests are clearly among the most important lignocellulosic sources, and the biorefineries can be developed based on current agro-forest industry facilities. One of the most discussed examples is the implementation of the biorefinery concept based on the existing pulp mills, producing added chemicals and other end-products from biomass residues and pulping waste streams, together with pulp and paper [2, 9-12].

1.1.3 Perspectives to the future

There are several requirements to the successful development of biorefineries in the future. Specific attention should be given to the development and application of industrial biorefinery technologies to become technically and economically viable [3, 9]. Furthermore the biorefining technologies will have to compete with oil based technologies, which have been optimised along the last century [4]. One important aspect is related with the logistic of the flow of feedstock's, being necessary to achieve ecological transport of biomass with reduced costs [1, 4]. However, one of the main drivers will be the use of sustainable and environmentally friendly principles, to separate, refine and transform biomass into energy, chemicals and materials. In fact, there is already a large research demand on the use of green chemical technologies in order to minimize the environmental footprints of the end-products [5, 9]. This

constitutes the main goal of the next generation biorefineries, in which the green chemistry will be a partner for a sustainable future.

1.2 Portuguese agro-forest industries to exploit within the biorefinery context

Portugal has in agro-forest industries one of the main economic sectors, with pulp and paper and cork industries playing a relevant position. Additionally, the pulp and paper industry is one of the most important industries in world; in fact about 300 million tonnes of paper and paperboard are produced worldwide every year [13]. *Eucalyptus* spp. are among the most important fiber sources for pulp and paper production [14], with around 19.5 million hectares of *Eucalyptus* spp. planted worldwide [15]. There are more than 600 species of *Eucalyptus*, however, only a few are relevant in terms of pulp and paper production. *E*. *globulus* is the predominant species planted in Portugal and Spain [16], while *E*. *grandis*, which has a tropical origin, is the main species planted in Brazil and South Africa [17]. Other species have been object of interest as raw material for pulp production, such as *E. maidenii* [18-20] and hybrids, as *E. urograndis* [21].

Cork, the outer bark of cork oak tree, *Quercus suber* L., is a unique material. Cork harvesting is a completely natural process, being cork a renewable, sustainable and biodegradable material. Furthermore, the cork oak forest has a great importance on the biodiversity of fauna and flora, being also defended that it has a key role on the protection of several endangered species [22]. Cork industry is one of the main economic sectors in Portugal. It corresponds about 1.7% of the employment and 1.8% of the gross value of production of the manufacturing sector [23]. Furthermore cork products are exported for more than 100 countries, representing about 158 000 tones of exports in 2010, which is equivalent to a value of 755 million Euros [22].

Both *Eucalyptus* pulping and cork industries generate substantial amounts of biomass residues, among which, bark, in pulp and paper industry, and cork powder and black condensate in cork industry, are the most abundant and are currently simply burned to produce energy. Therefore, the search for new applications is a key strategy in the up-grading of these residues, within the above described biorefinery concept [8]. In fact, there has been an increasing interest in the exploitation of these industrial by-products as a source or precursors of value-added renewable compounds [24-29].

The bark tissue of plants is rich in secondary metabolites, such as phenolic compounds, which have become in recent years one of the most studied groups of

natural components. Despite their numerous health benefits, one of the main interest in phenolic compounds is related with their antioxidant activity. The increasing interest in the extraction of phenolic compounds from biomass resources, and in particular from industrial by-products [30, 31], is related to two main goals: On one hand, as a way to up-grading industrial by-products and, on the other hand, as a response to the upcoming search to natural products. For instance, in the cosmetic industry, the current new trend is to return to the use of natural plant-derived products.

Obviously, the exploitation of industrial by-products as sources of valuable phenolic compounds has to start by a detailed study of that fraction, with a special concern with the extraction methodologies and techniques of chemical composition analysis used. The conjugation of environmentally friendly extraction procedures with the valorisation of low value biomass residues is of great interest both in the economical and environmental perspectives. Thus, hand in hand with the search of phenolic compounds from agro-forest residues, the use of new sustainable extraction techniques is also a basic requirement. Among several others, supercritical fluid extraction is becoming an increasingly important process to extract high value compounds. Furthermore this technology could be considered as a first step in a biorefinery, allowing to obtain valuable secondary metabolites, without affecting the bulk structure of the biomass feedstock [5, 32].

1.3 Aims and Scope

The detailed study of the chemical composition of pulp and paper and cork industries by-products is a key step towards the implementation of strategies for the recovery of valuable components from these biomass residues. Despite some information have already been reported concerning the phenolic fraction of cork [33-35] and *Eucalyptus globulus* bark [36-38], a complete study, mostly applying novel extraction and characterisation techniques of identification, has not yet been carried out.

In addition, no study has been done so far concerning the industrial cork by-products cork powder and black condensate, as well as, concerning barks from other important *Eucalyptus* species, such as *E. grandis*, *E. urograndis* and *E. maidenii*.

Finally, to our knowledge, no study has explored environmentally friendly extraction techniques of phenolic compounds in these biomass resources regarding a future industrial application. In this context several objectives were defined for this thesis:

- To explore the advantages of high-performance liquid chromatography and mass spectrometry in the identification and quantification of phenolic compounds;
- To select an appropriate method to analyse the antioxidant activity of the extracts obtained;
- To characterise the phenolic fractions of *E. globulus*, *E. grandis*, *E. urograndis* and *E. maidenii* barks;
- To optimise environmentally friendly extraction techniques, namely supercritical carbon dioxide (CO_2) in the extraction of phenolic compounds from *E. globulus* bark;
- To characterise the phenolic fraction of cork;
- Finally, to characterise the phenolic fraction of cork powder and black condensate from the cork processing industry.

1.4 Outline of this thesis

This thesis is organized in six chapters.

Following the introduction in **Chapter 1**:

Chapter 2 is a review of the most relevant literature, which includes a description of the cork and pulp and paper industries, evidencing the by-products generated there. A general description of *Eucalyptus* bark and cork composition is also presented (Part A). The second part of this chapter describes the existing different families of phenolic compounds, the main health benefits attributed to them, as well as a compilation of the different methodologies and techniques used in the analysis of phenolic compounds from vegetal sources (Part B).

Chapter 3 is divided in two parts. In Part A the study of the chemical composition of the phenolic fraction of *E. globulus* bark is presented. Two different conventional solid-liquid extraction conditions were used, and the respective analysis, involving the use of two different mass spectrometry techniques. Part B of this chapter is focused on the optimisation of supercritical CO_2 extraction of phenolic compounds from *E. globulus* bark. The optimal SFE conditions are also provided. The content of this chapter was adapted from two papers published in peer-reviewed journals.

Chapter 4 involves the study of the phenolic fraction of the bark from three other *Eucalyptus* species; *E. urograndis*, *E. grandis* and *E. maidenii*. The optimal solid-liquid extraction methodology achieved for *E. globulus* bark was applied here. Once more, the content of this chapter was adapted from a paper published in a peer-reviewed journal.

Chapter 5 describes the characterisation of phenolic fraction of cork as well as of the industrial cork by products, cork powder and black condensate. This chapter was divided in two parts. Part A concerns the analysis of the phenolic fraction of *Quercus suber* cork, using a previously described extraction methodology. A second extraction methodology was also applied. In Part B the extraction methodology used in *Eucalyptus* barks was applied to cork and related by-products, namely industrial cork powder and black condensate. The content of this chapter was adapted from a published paper (Part A) and from a submitted manuscript (Part B), both in peer-reviewed journals.

The adaptation of these chapters from articles involved the standardization of the formatting and nomenclature. Some sentences and images were added, in order to clarify some aspects. In some of the chapters a conclusion part was also added, when it was not part of the original paper.

Chapter 6 presents a general discussion of all the results obtained, representing not only a summary of the main results obtained as also a global comparative analysis of the different results. Final remarks and recommendations for future research activities are also presented in this chapter.

References

[1] Kamm, B.; Kamm, M.; Gruber, P. R.; Kromus, S., Biorefinery systems - An overview. In *Biorefineries - Industrial processes and products*, Kamm, B.; Gruber, P. K.; Kamm, M., Eds. Wiley-VCH: Weinheim, Germany, 2006; Vol. 1, pp 3-40.

[2] Kamm, B.; Kamm, M.; Schmidt, M.; Hirth, T.; Schulze, M., Lignocellulose-based chemical products and product family trees. In *Biorefineries - Industrial processes and products*, Kamm, B.; Gruber, P. R.; Kamm, M., Eds. Wiley-VCH: Weinheim, Germany, 2006; Vol. 2, pp 97-149.

[3] Ragauskas, A. J.; Williams, C. K.; Davison, B. H.; Britovsek, G.; Cairney, J.; Eckert, C. A.; Frederick, W. J.; Hallett, J. P.; Leak, D. J.; Liotta, C. L.; Mielenz, J. R.; Murphy, R.;

Templer, R.; Tschaplinski, T., The path forward for biofuels and biomaterials. *Science* **2006,** *311*, 484-489.

[4] Clark, J. H.; Deswarte, F. E. I.; (Eds.), The biorefinery concept - An integrated approach. In *Introduction to chemicals from biomass*, John Wiley & Sons, Ltd: Chichester, United Kingdom, 2008; pp 1-20.

[5] Clark, J. H.; Budarin, V.; Deswarte, F. E. I.; Hardy, J. J. E.; Kerton, F. M.; Hunt, A. J.; Luque, R.; Macquarrie, D. J.; Milkowski, K.; Rodriguez, A.; Samuel, O.; Tavener, S. J.; White, R. J.; Wilson, A. J., Green chemistry and the biorefinery: A partnership for a sustainable future. *Green Chem.* **2006,** *8*, 853-860.

[6] Gallezot, P., Process options for converting renewable feedstocks to bioproducts. *Green Chem.* **2007,** *9*, 295-302.

[7] National Renewable Energy Laboratory. What is a biorefinery? http://www.nrel.gov/biomass/biorefinery.html (October 12, 2012)

[8] Fernando, S.; Adhikari, S.; Chandrapal, C.; Murali, N., Biorefineries: Current status, challenges, and future direction. *Energ. Fuel* **2006,** *20*, 1727-1737.

[9] Clark, J. H., Green chemistry for the second generation biorefinery - sustainable chemical manufacturing based on biomass. *J. Chem.Technol. Biotechnol.* **2007,** *82*, 603-609.

[10] Van Heiningen, A., Converting a kraft pulp mill into an integrated forest biorefinery. *Pulp Pap.-Can.* **2006,** *107*, 38-43.

[11] Mao, H.; Genco, J. M.; van Heiningen, A.; Pendse, H., Kraft mill biorefinery to produce acetic acid and ethanol: Technical economic analysis. *BioResources* **2010,** *5*, 525-544.

[12] Huang, H.-J.; Ramaswamy, S.; Tschirner, U. W.; Ramarao, B. V., A review of separation technologies in current and future biorefineries. *Sep. Purif. Technol.* **2008,** *62*, 1-21.

[13] Smook, G. A., *Handbook for pulp & paper technologists*. 3rd ed.; Angus Wilde Publications Inc.: Vancouver, 2002.

[14] Rencoret, J.; Gutierrez, A.; del Rio, J. C., Lipid and lignin composition of woods from different eucalypt species. *Holzforschung* **2007,** *61*, 165-174.

[15] Trabado, G. I.; Wilstermann, D. Cultivated *Eucalyptus* Forests Global Map 2008 Version 1.0.1. http://www.git-forestry.com (September 11, 2012)

[16] Potts, B. M.; Vaillancourt, R. E.; Jordan, G.; Dutkowski, G.; Silva, J.; McKinnon, G.; Steane, D.; Volker, P.; Lopez, G.; Apiolaza, L.; Li, L. N.; Marques, C.; Borralho, N. In *Eucalyptus in a changing world*, IUFRO Conference, Aveiro, Portugal, 11-15 October, 2004; Borralho, N.; Pereira, J. S.; Marques, C.; Coutinho, J.; Madeira, M.; Tomé, M., Eds. Aveiro, Portugal, 2004.

[17] Ugalde, L.; Pérez, O. Mean annual volume increment of selected industrial forest plantation species. www.fao.org/forestry (September 28, 2012)

[18] Kibblewhite, R. P.; Johnson, B. I.; Shelbourne, C. J. A. In *Kraft pulp qualities of Eucalyptus nitens, E. globulus, and E. maidenii, at ages 8 and 11 years*, 55th Appita Annual Conference Proceedings, Hobart, Australia, 2001; Hobart, Australia, 2001.

[19] Lopez, G.; Potts, B.; Rodriguez, J. T.; Gelid, P. In *The performance of Eucalyptus maidenii provenances in Argentina*, IUFRO International symposium-Developing the Eucalyptus of the Future Valdivia, Chile, 10-15 September, 2001; Valdivia, Chile, 2001.

[20] Li, D. Q.; Liu, Y. P.; Zheng, H. S.; Zhu, R. G.; Huang, Y. X., Analysis of genetic effects on growth traits in Eucalyptus *maidenii* F. Muell. In *Eucalyptus Plantations: Research , Management and Development*, Wei, R. P.; Xu, D., Eds. World Scientific: Singapore, 2003; pp 229-238.

[21] Quilhó, T.; Miranda, I.; Pereira, H., Within-tree variation in wood fibre biometry and basic density of the urograndis eucalypt hybrid (*Eucalyptus grandis* x *E. urophylla*). *IAWA J.* **2006,** *27*, 243-254.

[22] APCOR (Associação Portuguesa da Cortiça). 2011 Yearbook. http://www.realcork.org/userfiles/File/Publicacoes/anuario2011.pdf (September 19, 2012)

[23] Fortes, M. A.; Rosa, M. E.; Pereira, H., *A cortiça*. IST Press: Lisbon, 2004.

[24] Gandini, A.; Pascoal Neto, C.; Silvestre, A. J. D., Suberin: A promising renewable resource for novel macromolecular materials. *Prog. Polym. Sci.* **2006,** *31*, 878-892.

[25] Pinto, P. C. R. O.; Sousa, A. R.; Silvestre, A. J. D.; Neto, C. P.; Gandini, A.; Eckerman, C.; Holmbom, B., Quercus suber and Betula pendula outer barks as renewable sources of oleochemicals: A comparative study. *Ind. Crop. Prod.* **2009,** *29*, 126-132.

[26] Sousa, A. F.; Pinto, P. C. R. O.; Silvestre, A. J. D.; Neto, C. P., Triterpenic and other lipophilic components from industrial cork byproducts. *J. Agric. Food Chem.* **2006,** *54*, 6888-6893.

[27] Domingues, R. M. A.; Sousa, G. D. A.; Freire, C. S. R.; Silvestre, A. J. D.; Pascoal Neto, C., *Eucalyptus globulus* biomass residues from pulping industry as a source of high value triterpenic compounds. *Ind. Crop. Prod.* **2010,** *31*, 65-70.

[28] Vazquez, G.; Gonzalez-Alvarez, J.; Santos, J.; Freire, M. S.; Antorrena, G., Evaluation of potential applications for chestnut (*Castanea sativa*) shell and eucalyptus (*Eucalyptus globulus*) bark extracts. *Ind. Crop. Prod.* **2009,** *29*, 364-370.

[29] Domingues, R. M. A.; Patinha, D. J. S.; Sousa, G. D. A.; Villaverde, J. J.; Silva, C. M.; Freire, C. S. R.; Silvestre, A. J. D.; Neto, C. P., *Eucalyptus* biomass residues from agro-forest and pulping industries as sources of high-value triterpenic compounds. *Cell Chem. Technol.* **2011,** *45*, 475-481.

[30] Balasundram, N.; Sundram, K.; Samman, S., Phenolic compounds in plants and agri-industrial by-products: Antioxidant activity, occurrence, and potential uses. *Food Chem.* **2006,** *99*, 191-203.

[31] Moure, A.; Cruz, J. M.; Franco, D.; Domínguez, J. M.; Sineiro, J.; Domínguez, H.; José Núñez, M. a.; Parajó, J. C., Natural antioxidants from residual sources. *Food Chem.* **2001,** *72*, 145-171.

[32] Deswarte, F. E. I.; Clark, J. H.; Wilson, A. J.; Hardy, J. J. E.; Marriott, R.; Chahal, S. P.; Jackson, C.; Heslop, G.; Birkett, M.; Bruce, T. J.; Whiteley, G., Toward an integrated straw-based biorefinery. *Biofuel Bioprod. Bior.* **2007,** *1*, 245-254.

[33] Conde, E.; Cadahía, E.; García-Vallejo, M. C.; de Simón, B. F.; Adrados, J. R. G., Low molecular weight polyphenols in cork of *Quercus suber*. *J. Agric. Food Chem.* **1997,** *45*, 2695-2700.

[34] Conde, E.; Cadahia, E.; Garcia-Vallejo, M. C.; de Simon, B. F., Polyphenolic composition of *Quercus suber* cork from different Spanish provenances. *J. Agric. Food Chem.* **1998,** *46*, 3166-3171.

[35] Conde, E.; Cadahía, E.; García-Vallejo, M. C.; González-Adrados, J. R., Chemical characterization of reproduction cork from spanish *Quercus suber*. *J. Wood Chem. Technol.* **1998,** *18*, 447-469.

[36] Conde, E.; Cadahía, E.; García-Vallejo, M.; Tomás-Barberán, F., Low molecular weight polyphenols in wood and bark of *Eucalyptus globulus Wood Fiber Sci.* **1995,** *27*, 379-383.

[37] Conde, E.; Cadahia, E.; DiezBarra, R.; GarciaVallejo, M. C., Polyphenolic composition of bark extracts from *Eucalyptus camaldulensis*, *E. globulus* and *E. rudis*. *Holz Als Roh- und Werkst.* **1996,** *54*, 175-181.

[38] Cadahía, E.; Conde, E.; deSimon, B. F.; GarciaVallejo, M. C., Tannin composition of *Eucalyptus camaldulensis*, *E. globulus* and *E. rudis*. Part II. Bark. *Holzforschung* **1997,** *51*, 125-129.

Chapter 2

Bibliographic review

Part A

Quercus suber L. cork and *Eucalyptus* bark

2.1 Cork and pulp and paper industries

Portugal has in agricultural and forest domains one of the main economic sectors. Besides the dimension of the country, 39% of the area of Portugal is occupied by forest, corresponding about 3 500 000 hectares [1]. Most of this area is occupied by pine, cork oak and eucalyptus plantations (Figure 2.1).

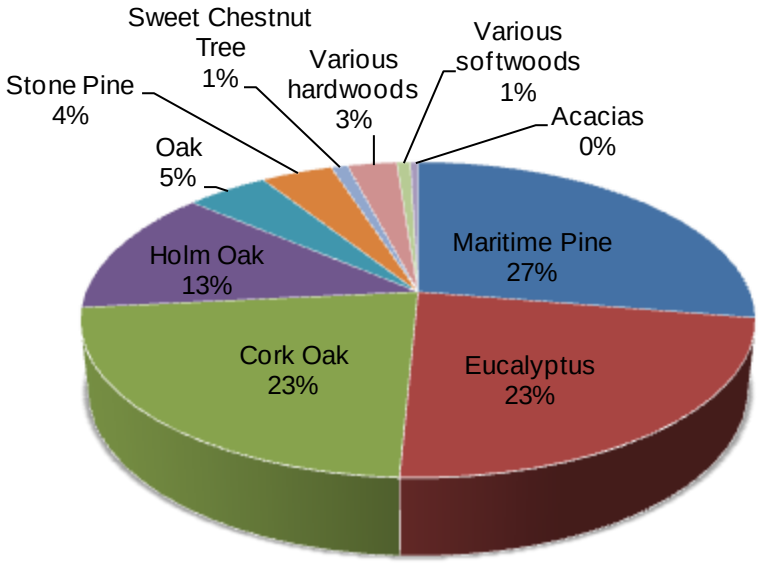


Figure 2.1 – Representative scheme of the forestry occupation by different tree classes in Portugal (source [1])

These trees are the main raw materials of two of the most important industries of the country: the cork and the pulp and paper industries.

2.1.1 *Eucalyptus* and pulp and paper processing

Pulp, mainly composed of cellulose fibers, is the raw material for paper production and has its origin mostly in plants and particularly in hardwood trees, due to its availability, growing feature and economical and technical factors. Between them, *Eucalyptus* species are the main fiber sources, due to their fast growing, short rotation periods and favourable pulping and bleaching ability [2]. *Eucalyptus* plantation areas cover about 19.5 million hectares worldwide [3]. Most of the planted eucalypts are from the subgenus Symphyomyrtus, sections Latoangulatae (*E. grandis E. urophylla*), Maidenaria (*E. globulus*, *E. nitens*) and Exsertaria (*E. camaldulensis*, *E. tereticornis*), including hybrids of some of these species [4].

Eucalyptus globulus Labill. (Figure 2.2) is the main raw material for pulp and paper industries in Portugal and Spain [5]. There is about 672 000 hectares of *E. globulus* planted in Portugal, ranking the third in terms of forest area and representing about 31% of the world production of *E. globulus* [5].

Figure 2.2 – Illustration of *Eucalyptus* trees, from left to right: *E. globulus*, *E. grandis*, *E. urograndis* and *E. maidenii*

E. grandis (Figure 2.2) is the most cultivated specie for industrial purposes, particularly in South Africa and Brazil [2]. Due to its pulping, bleaching and papermaking properties, this specie is one of the main used as fiber source not only in those countries but also in Congo and China [6]. Furthermore, due to its genetic characteristics it is also commonly used in the development of hybrids.

E. urograndis (Figure 2.2), which is a hybrid between *E. grandis* and *E. urophylla*, is produced in Brazil, being developed to conjugate the high density and superior pulp properties of *E. urophylla* wood and fast growing properties of *E. grandis* [7]. This demonstrates the increasing interest on the exploitation of *Eucalyptus* spp. as raw material for pulp production and papermaking in the South America. Actually, during the last 5 years, the eucalyptus forest area in Brazil has increased 5.3% per year, becoming the 6th world pulp producer in 2010 [8].

E. maidenii (Figure 2.2) is, at the moment, not so commonly used as a fiber source for pulp production as others *Eucalyptus* species, however, its potential for forest developing and excellent pulp qualities has also been demonstrated [9]. In fact, several countries, as Argentina [10] or even China [11] have shown an increasing interest in to considerer *E. maidenii* fibers as an alternative raw material for pulp production.

The pulp industries generate substantial amounts of biomass residues, among which bark is the most abundant and is currently simply burned to produce energy. In the case of *E. globulus*, bark represents about 11% of the stem dry weight [12]. Thus, a pulp mill with a production capacity of $5.0x10^5$ tonnes/year of bleached kraft pulp can generate around $1.0x10^5$ tonnes/year of bark, showing the enormous potential for the upgrading of this biomass residue. Apart from the bark residues produced in the mill, large amounts of other biomass residues such as leaves, branches and fruits, resulting from logging operations, are also burned in biomass boilers for energy production or are simply left in the forest for soil nutrition.

2.1.2 Cork, cork oak and cork processing

Cork is the outer bark of *Quercus suber* L. (Figure 2.3), which is a native tree from Mediterranean region, and occupies in Portugal about 23% of the forestry area, representing more than 700 000 ha [1, 13]. Due to its characteristic climate, dry summers and mild winters, Alentejo is the region with the highest cork oak forestland concentration (about 72% of the total area occupied by this species in Portugal) [13].

Q. suber is harvested at a minimum legal periodicity of 9 years [14]. The unique properties of this species are related with its capacity to regenerate bark, forming new cork layers. The first cork removing (virgin cork) is only made in trees with 15-30 years old, and each cork oak could reach about 200 years old. The higher quality of cork is only achieved after the third harvesting, from which the cork is called "*amadia*" grade.

Cork presents very interesting and unique properties, such as a low density (with values between 120 and 180 Kg m^{-3} for *amadia* grade), hydrophobic character, viscoelastic behaviour and thermal, acoustic and electric insulation properties [15-17].

Portugal is the main exporter of cork based products in the world (53%), resulting from the activity of about 700 industries of this sector, 75% of which located in Aveiro region (Santa Maria da Feira).

The main products of the cork processing are the well-known cork stoppers (Figure 2.3) for wine and champagne bottles, representing about 40% of the production process [13]. However, due to its physical and mechanical properties described above, there are a large number of other cork applications. Several types of agglomerates, insulation cork boarding, flooring and walls coverings or rubber/cork based agglomerates are some of other products from cork processing [14, 16]. In the last decade there was also a high

demand for the use of cork for furniture, footwear, clothe and even some high-tech in aeronautics applications.

Figure 2.3 – a) Cork oak forest, known as "montado" b) cork oak tree; c) cork planks d) cork stoppers (source [13])

During cork processing, several residues are generated, called industrial cork powder, black condensate and cooking wastewaters. A representative scheme of the industrial cork processing is illustrated in Figure 2.4. Cork powder, the most abundant cork by-product, represents in Portugal about 34 000 ton per year [18]. This residue is generated during the trituration process to obtain cork granulates (Figure 2.4), which uses the wastes generated during the cork stoppers production, virgin cork and low quality *amadia* cork [16]. The low particle size of cork powder (less than 0.25 mm) do not allow its use in current industrial uses, even in the agglomerates production, which would entail the use of high amounts of adhesives to cope with its high specific area [16, 18].

Some studies were already developed concerning valorisation of industrial cork powder, such as in the production of composites [16] or in environmental adsorption technologies [19], however, none of these has been industrialized. Consequently, this residue has so far a low commercial value, being mostly used in the biomass boilers to produce energy [18].

Black condensate results from the insulation corkboard production process (Figure 2.4), which uses black agglomerates at high pressures and temperatures in the range of 250-

500 ºC without any adhesive [18]. During this process vapors are formed, which after cooling generate a waxy solid waste in autoclave pipes. This residue is collected periodically, in amounts of about 2 100 tonnes per year, being also burned to produce energy [18]. Cooking wastewaters, another cork residue, is obtained as a liquid effluent during the cooking of the cork in boiling water.

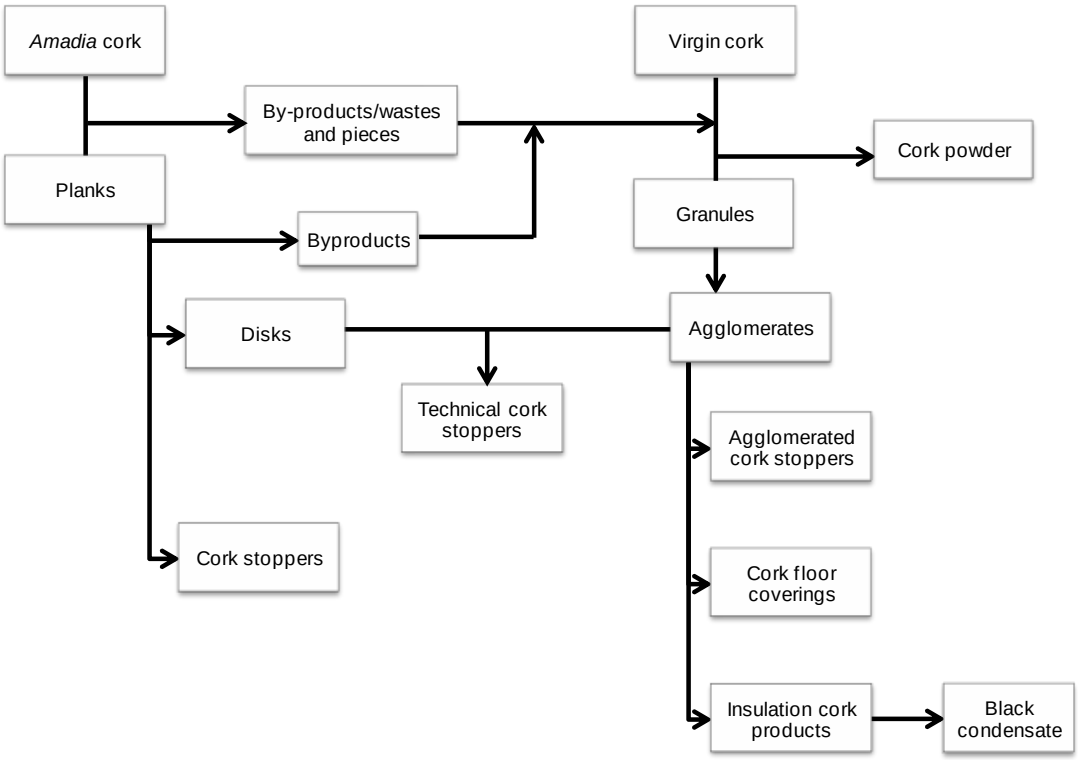


Figure 2.4 – Representative scheme of the industrial cork processing (adapted from [16])

2.2 Cork and *Eucalyptus* bark composition

2.2.1 General barks structure

The bark, which protects the trees against physical and biological attacks, constitutes the outer part of woody stem, branches and roots and can be divided in inner and outer bark [20, 21]. The formation of bark is initialised by the cell division of cambium, where the growth of tree occurs. Here xylem is produced, in the woody side and phloem, also known as the inner bark. This is a narrow tissue in which the sap with carbohydrates moves up and down through sieve tubes and rays, transporting the nutrients between the leaves,

needles or roots and the stem and branches. The outer bark is a dead tissue, whose cells already existed in the inner bark [20, 22].

The study of the chemical composition of barks is a complex task, due to its variability between tree species as within the morphological parts of the tree. The cell walls of bark, as in wood, are divided in structural and non-structural components (Figure 2.5).

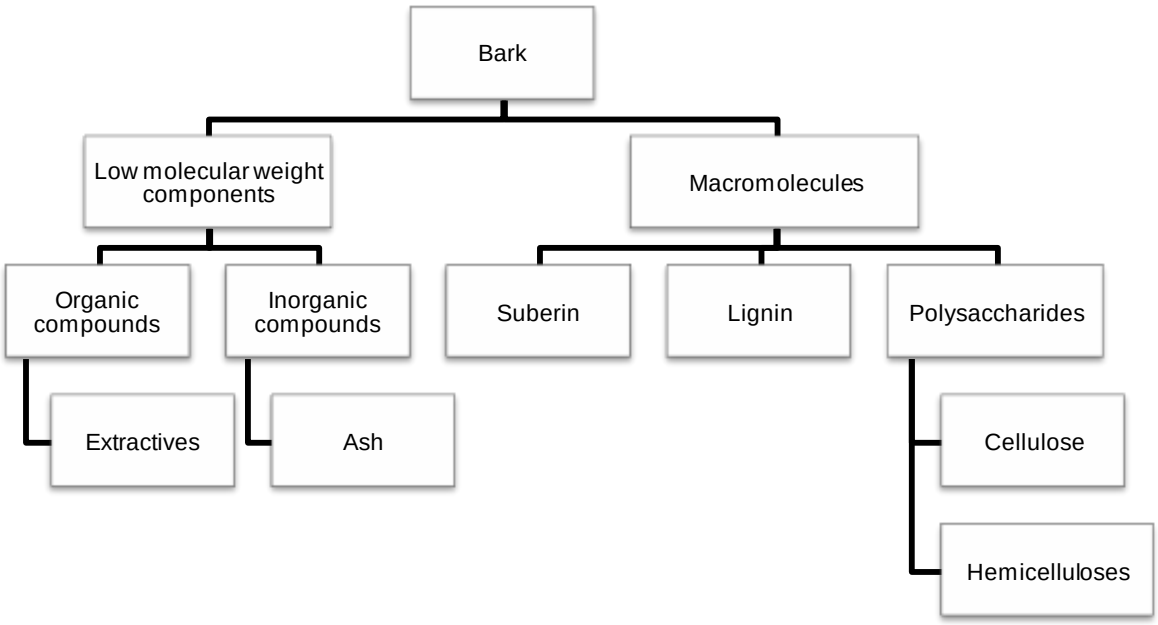


Figure 2.5 – General chemical composition of barks

The structural components comprise polysaccharides (cellulose and hemicelluloses) and lignin. Cellulose is a linear homopolymer with glucose units linked by glycosidic bonds and could represent about 40-45% of the wood of most species, while in the bark this value decreases to around 20% [22]. Lignin confers rigidity and resistance to the impact and compression [20]. Furthermore bark has another structural component, suberin, which is not present in wood. This is composed by an aromatic-aliphatic cross-linked polyester, which plays an important role on thermal and hydric insulation of bark tissues [23].

The non-structural components of bark, which are soluble compounds, are divided into inorganic and organic components, known as ash and extractives, respectively. These components are generally present in higher amounts in bark than in wood [20]. The inorganic constituents (determined as ash) are metallic salts, predominantly of calcium, potassium and magnesium, while the extractives include aliphatic compounds (fatty acids

and alcohols and hydrocarbons), terpenic and phenolic compounds, among other families [20]. The role of these components in the tree is related to protection against herbivores or plagues attacks [24]. Therefore, apart from bark, this group of components can usually only be found along wood in the heartwood section [22].

2.2.2 *Eucalyptus* bark composition

The chemical composition of the bark from *E. globulus* concerning the structural and non-structural components has been object of a limited number of studies. The *E. globulus* bark composition (Table 2.1) varies among samples, which could be related with the different origins, age of the tree or morphological part from which the bark was harvested. Only recently the content of suberin of *E. globulus* bark was reported [25, 26], which is about 1%. Miranda *et al.* [27] reported that the cellulose content of *E. globulus* bark (56.0%) is quite similar to wood (56.9%). In fact, the main difference between the two tissues is the extractives content: the bark showed extractives content two times higher than wood [27]. Controversially, earlier Pereira *et al.* [28] have reported very similar extraction yield values for wood and bark (around 8%).

Table 2.1 – Summary of the global chemical composition (%) of *E. globulus, E. grandis* and *E. urograndis* barks

	Suberin	Lignin	Polysaccharides	Extractives	Ash	Source
E. globulus	-	22.3	62.8	8.0	1.0	[28]
	-	16.7	62.5	12.4[a]	4.74	[29]
	1.0	22.3	67.2	7.2	12.8	[25]
	-	16.9	79.7	6.6	2.9	[27]
	1.0	26.6	62.6	6.5	12.1	[26]
E. grandis	-	11.4	51.0	26.6	7.1	[30]
E. urograndis	-	16.9	50.8	25.8	4.1	[30]

[a] sum of the extraction yields with n-hexane, ethanol, methanol and water

It is worth mentioning the fact that the polar extractive components in *E. globulus* bark are more abundant than the non-polar components. Mirra [25] reported extraction yields of about 4.58% and 1.43% with water and ethanol solid-liquid extractions, respectively, while with dichloromethane only an extraction yield of 0.96% was achieved. Vázquez *et al.* [29]

also reported a low extraction yield with n-hexane (0.42%), whilst with polar solvents, such as water or a methanol/water mixture, the extraction yield reaches values of 6.80 and 5.19%, respectively. A difference in the lipophilic content of the extractives components between the inner and outer bark of *E. globulus* was reported [31]. Concerning the inorganic non-structural components, Mirra [25] described a content of about 12.8%, however, most of the studies reported ash content values lower than 5% [27-29].

Information about the contents of structural and non-structural components of *E. grandis*, *E. urograndis* and *E. maidenii* barks is extremely scarce. In fact, only Bargatto reported, in his thesis [30], the global chemical composition of *E. grandis* and *E. urograndis*. Lower values of polysaccharides and lignin were achieved, comparing with *E. globulus* composition (Table 2.1). An extremely high value of extractives content was reported for these two species, 26.6% and 25.8% for *E. grandis* and *E. urograndis*, respectively [30].

Regarding the chemical composition of the bark from *E. grandis*, *E. urograndis* and *E. maidenii*, this is also largely an unexplored topic. In fact, only information concerning the lipophilic fraction of the non-structural extractives components, obtained with dichloromethane, has been found [32]. However, this information is enough to evidence the differences in the chemical composition between these species. Notwithstanding, variations in the lipophilic content between the inner and outer barks of those species have also been observed. *E. maidenii*, both in the inner and outer bark, has a higher lipophilic content than *E. globulus* [31], *E. grandis* and *E. urograndis,* with values of 2.6 and 6.1%, respectively [32]. *E. grandis* and its hybrid *E. urograndis* have lipophilic extraction yields in the same range, with values in the inner bark of 0.3 and 0.5% and in the outer bark of 1.7 and 1.3%, in that order [32]. These two species present a lipophilic content in the outer bark higher than *E. globulus*, however, in the inner bark the values are similar [31, 32].

The extractives components of the bark of *Eucalyptus* spp., together with its colour, are the main responsible of the disadvantages in the use of this tissue of the tree in the pulp production [22].

2.2.2.1 Polysaccharides

Polysaccharides comprise mainly cellulose and hemicelluloses in woody materials. Cellulose is a linear macromolecule composed by $\beta(1\rightarrow4)$ linked D-glucopyranose units (Figure 2.6) [20, 23]. Cellulose contents range between 41.6 [29] and 54.9% [28] in *E. globulus* bark.

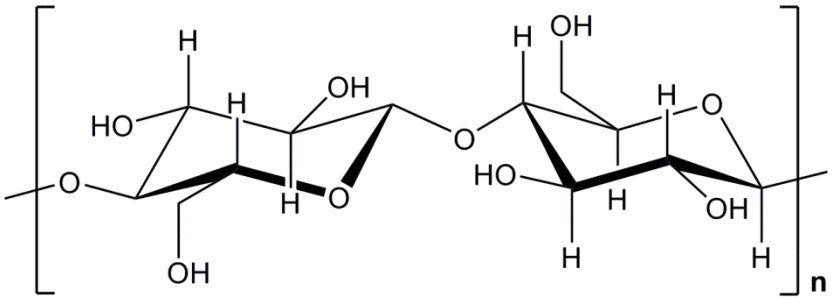


Figure 2.6 – Cellulose structure

Hemicelluloses are heteropolymers composed by different monosaccharides (Figure 2.7), including pentoses (xylose, arabinose) and hexoses (glucose, mannose, galactose, rhamnose, glucuronic acid), sometimes with functionalities, such as methyl or acetyl groups. Furthermore, hemicelluloses have a degree of polymerization lower than cellulose and branched chains [20, 23].

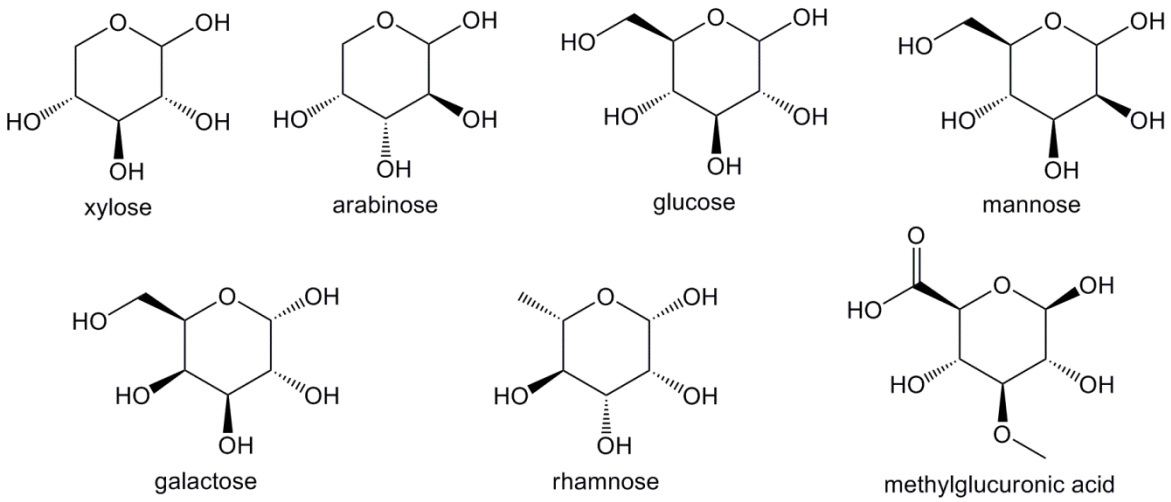


Figure 2.7 – Polysaccharide repetitive monomers

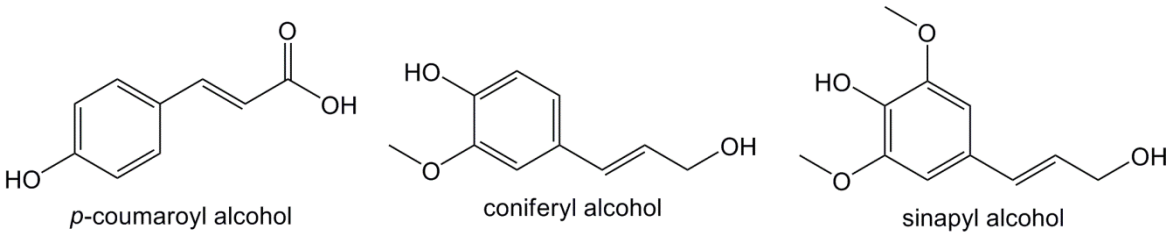
The polysaccharides content of *E. globulus* bark ranges between 62.5 [29] and 79.7% [27], being this fraction composed mainly by glucose and xylose (Table 2.2). Bargatto [30] reported the polysaccharides composition of *E. grandis* and *E. urograndis* with glucose contents of 77.6 and 76.4% and xylose contents of 16.9 and 18.9%, respectively (Table 2.2). Taking the ratio between glucose and xylose as indicative of cellulose to hemicelluloses, there is a significative difference between the three species: a ratio about 3 for *E. globulus* against 4.58 for *E. grandis* and 4.04 for *E. urograndis*. No information has been found about the polysaccharides content of *E. maidenii* bark.

Table 2.2 – Relative monosaccharides content (%) of *E. globulus*, *E. grandis* and *E. urograndis* barks

Monosaccharides	***E. globulus***		***E. grandis***	***E. urograndis***
	[29]	[26]	[30]	[30]
Glucose	70.4	68.4	77.6	76.4
Mannose	1.4	1.9		
Galactose	3.6	3.3	2.3	1.8
Rhamnose	-	0.4	0.7	0.6
Xylose	20.8	23.2	16.9	18.9
Arabinose	3.7	2.7	2.2	2.0

2.2.2.2 Lignin

Lignin is a polyphenolic cross-linked and amorphous polymer built up from phenylpropane units, such as *p*-coumaroyl, coniferyl and synapil alcohols, which are linked by ether and carbon-carbon bonds (Figure 2.8). The aromatic core of these units is denominated by *p*-hydroxyphenyl, guaiacyl and syringyl units, respectively [20].



p-coumaroyl alcohol coniferyl alcohol sinapyl alcohol

Figure 2.8 – Lignin precursors

The relative abundance of each unit is dependent of each species. To our knowledge, no study concerning the lignin composition of *Eucalyptus* spp. bark has been published so far.

2.2.2.3 Suberin

Suberin consists of a cross-linked amorphous polyester structure with long chain fatty acids, hydroxy fatty acids and phenolic compounds, linked by esters bonds [33, 34]. The detailed composition of the monomeric composition of suberin, as well as how the monomeric units are assembled at a macromolecular level, is not yet completely understood. Moreover, in the case of *Eucalyptus* bark, the analysis of suberin remains an unexploitable field. Actually, only recently the suberin content for *E. globulus* bark was reported [26, 27] and corresponds to a particularly lower value of the bark composition

(about 1%). However, Freire *et al.* [31] detected several fatty acids and alcohols as well as ω-hydroxy fatty acids, most of them with more than 20 carbon atoms, after an alkaline hydrolysis of dichloromethane extracts of *E. globulus* bark. It was reported that the presence of these esterified components could be related to the suberin-type structure [31]. In the same way, it was verified that 67% of fatty acids and 50% of aliphatic alcohols detected in the dichloromethane extracts of *E. grandis*, *E. urograndis* and *E. maidenii* outer barks are in esterified forms [32].

2.2.2.4 Inorganic components

The analysis of inorganic components of vegetal sources is done after the combustion of the material and commonly by elemental analysis. Miranda and co-workers [26] detected high amounts of zinc, chromium and nickel in the ash fraction of *E. globulus* bark.

To our knowledge, no study has been reported concerning the analysis of inorganic components of *E. grandis*, *E. urograndis* and *E. maidenii*.

2.2.2.5 Extractives

The extractives fraction of *E. globulus* bark can be divided mainly into a lipophilic and phenolic fraction. The lipophilic fraction is commonly extracted with dichloromethane and is composed by fatty acids, aliphatic alcohols, triterpenic compounds and sterols.

Freire *et al.* [31] analysed the lipophilic fraction of *E. globulus*, comparing the inner and outer fractions of bark. Outer bark presents lipophilic extractive contents about nine times higher than the inner bark. The outer fraction of *E. globulus* bark extractives is particularly rich in triterpenic compounds and mostly in triterpenic acids, such as ursolic, betulinic and oleanolic acids (Figure 2.9) [31]. Domingues *et al.* [35] reported a triterpenic content of the surface layers of *E. globulus* bark of about 32.3 g Kg^{-1}. Outer bark also contains sesquiterpene type compounds. Furthermore, Yun *et al*. [36] also reported the existence of monoterpenes in *E. globulus* bark.

Inner bark is particularly rich in sterols, among which *β*-sitosterol (Figure 2.9) and *β*-amirine are the major compounds [31, 37]. Fatty acids and alcohols are minor components of both inner and outer barks, being palmitic (Figure 2.9), oleic and linoleic acids and docosanol (Figure 2.9), tetracosanol and hexacosanol the main components of these families. Furthermore, α-hydroxy fatty acids, such as 2-hydroxypentacosanoic (Figure 2.9) and 2-hydroxytetracosanoic acids, are also present in the extractives from both inner and outer barks of *E. globulus* [31].

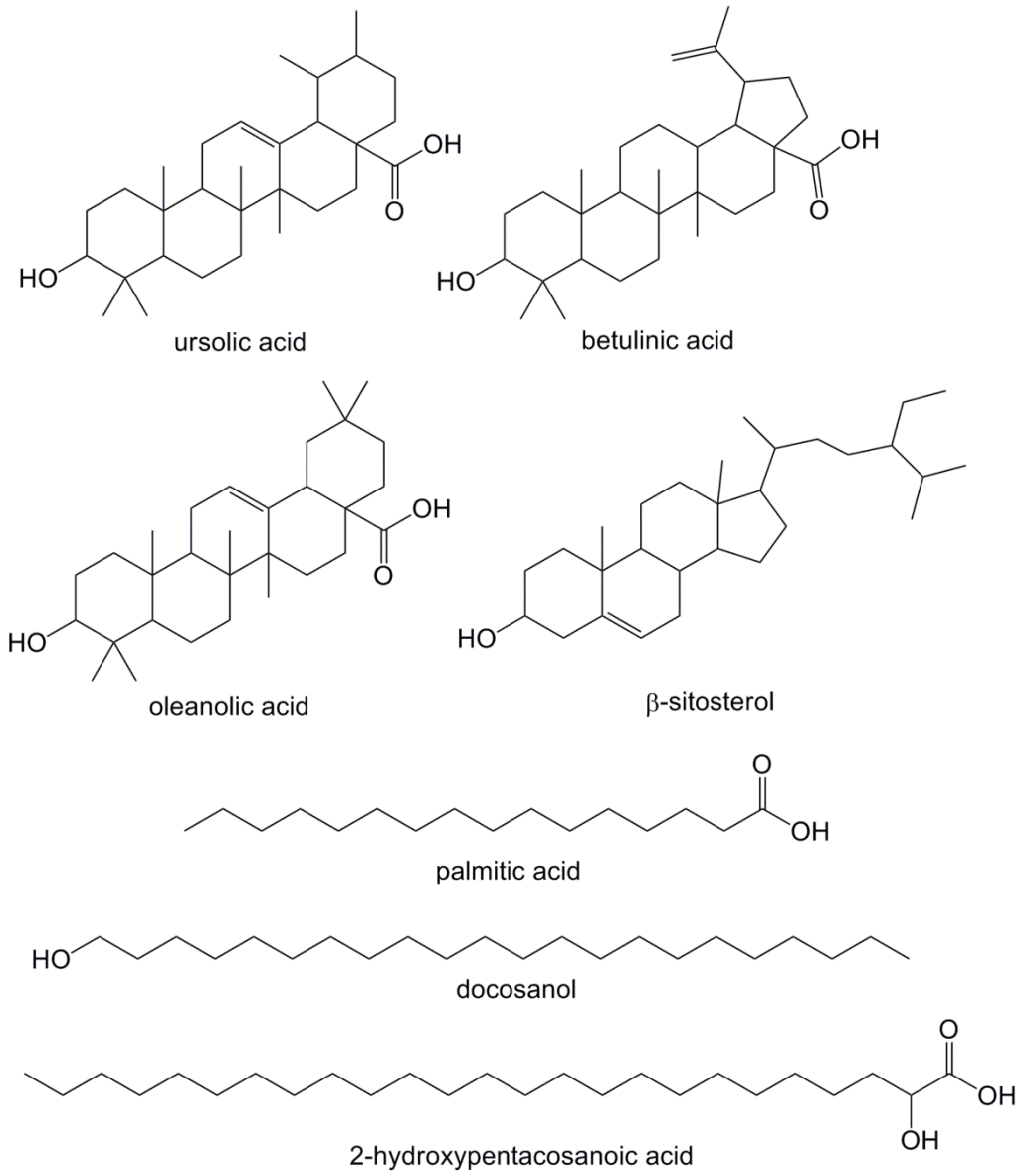


Figure 2.9 – Major lipophilic components of *E. globulus* bark

The lipophilic composition of *E. grandis*, *E. urograndis* and *E. maidenii* barks has also been studied [32]. Triterpenic acids (ursolic, oleanolic and linoleic acids) are, like for *E. globulus*, the major components of the outer barks of these species. However, these compounds are in lower amounts than in *E. globulus*. *β*-sitosterol is also present in high amounts, followed by minor quantities of fatty acids and long chain aliphatic alcohols. The inner barks of these two species also present high amounts of triterpenoids and sterols. In opposition, the lipophilic fraction of both the inner and outer barks of *E. maidenii* is mostly composed by monoterpenes and sesquiterpenes [32].

The other important group of extractives of *Eucalyptus* bark mentioned above corresponds to phenolic compounds. These are aromatic compounds with hydroxyl groups, and, according to their structure, can be divided in several families, such as phenolic acids and aldehydes, cinnamic acids, flavonoids, condensed and hydrolysable tannins, among others. A detailed description of the structural characteristics of each group of compounds is done in the part B of this chapter.

Most of the studies concerning the analysis of phenolic fraction of *E. globulus* bark have been carried out using a methanol:water mixture as extraction solvent [38]. Extractions with methanol [39] or alkaline solutions [40] were also applied. It is already known that the phenolic fraction of *E. globulus* comprises a wide range of compounds (Table 2.3).

Ellagic, gallic and protocatechuic acids (Figure 2.10) are reported to be the major phenolic acids present in *E. globulus* bark [38]. This tissue of *E. globulus* also presents high amounts of flavonoids, among which eriodictyol and taxifolin (Figure 2.10) are the major components. However, the main fraction of phenolic compounds in *E. globulus* bark is described to correspond to ellagitannins [38, 41]. Ellagitannins, which belong to hydrolysable tannins class of phenolic compounds, are polyphenols with a carbohydrate core linked to complex derivatives of ellagic acid. Several methyl and glycosyl derivatives of ellagic acid (Figure 2.10) have been detected in methanol extracts of *E. globulus* bark [39, 42].

The presence of several gallotannins in *E. globulus* bark has also been described. Gallotannins are analogous to ellagitannins but with the carbohydrate core linked to gallic acid units. Gallotannins from monogalloylglucose (Figure 2.10) to tetragalloylglucose were detected in *E. globulus* bark [40]. Conde and co-workers reported that *E. globulus* is characterised also by the presence of high amounts of proanthocyanidins [43], which are polymers composed of catechin units (Figure 2.10). In fact, Yazaki and Hills have already described the presence of catechin like polymers in *E. globulus* bark [39].

Table 2.3 – Phenolic compounds previously identified in *E. globulus* bark extractives

Phenolic compounds	**Source**
Pyrogallol	[44]
Protocatechuic aldehyde	[38, 41]
Protocatechuic acid	[38, 41]
Vanillic acid	[38, 41]
Gallic acid	[38, 39, 41, 44]
Methyl gallate	[36]
Apigenin	[41]
Naringenin	[38, 41]
Eriodictyol	[36, 38, 41]
Catechin	[36, 39, 40, 44]
Epicatechin	[40]
Ellagic acid	[38-41, 44]
Quercetin	[36, 38, 41]
Taxifolin	[36, 38, 41]
Trimethylellagic acid	[44]
Chlorogenic acid	[39]
Pinoresinol	[36]
Rhamnetin	[36]
Isorhamnetin	[40]
Rhamnazin	[36]
Engeletin	[36]
Gallocatechin	[39]
Quercetin-3-*O*-rhamnoside	[40]
Galloyl-glucose	[40]
Digalloyl-glucose	[40]
Trigalloyl-glucose	[40]
Tetragalloyl-glucose	[40]
3-methylellagic acid-rhamnoside	[39]
3-methylellagic acid-glucoside	[39]
3-*O*-methylellagic acid 3'-*O*-*α*-rhamnopyranoside	[42]
3-*O*-methylellagic acid 3'-*O*-*α*-2''-*O*- α-acetylrhamnopyranoside	[42]
3-*O*-methylellagic acid 3'-*O*-*α*-3''-*O*- α-acetylrhamnopyranoside	[42]
3-*O*-methylellagic acid 3'-*O*-*α*-4''-*O*- α-rhamnopyranoside	[42]
3,4,5-trimethoxyphenol 1-*O*-*β*-D-(6'-*O*-galloyl)glucopyranoside	[36]

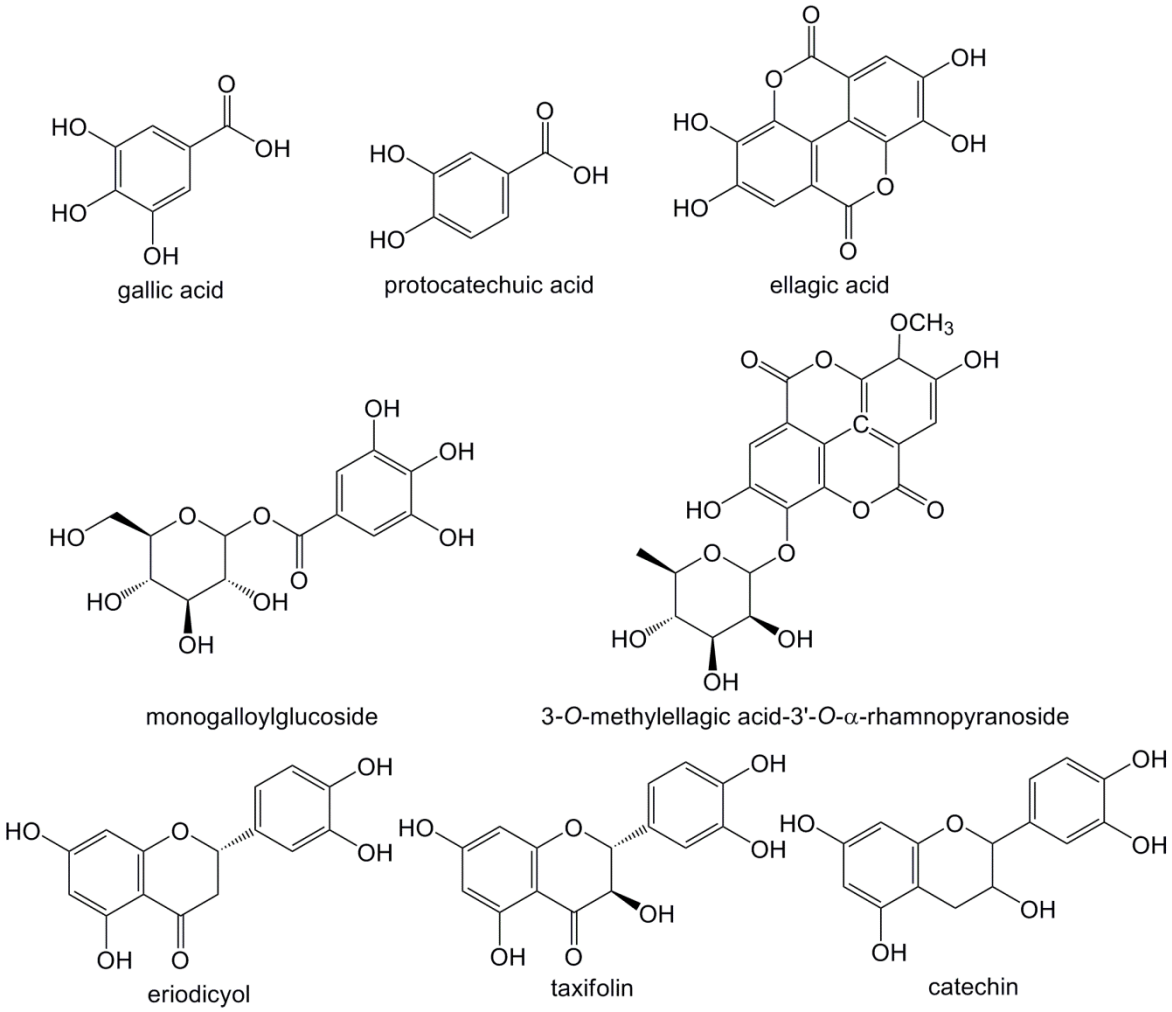


Figure 2.10 – Major phenolic compounds previously identified in *E. globulus* bark extractives

To our knowledge, there is no information about the phenolic fraction of *E. grandis*, *E. urograndis* and *E. maidenii* barks.

2.2.3 Cork composition

Contrary to *Eucalyptus* bark, the composition of *Q. suber* bark has been extensively studied, mainly due to the numerous applications of this bio-resource. Furthermore, the unique properties of cork are due to its unusual cellular structure and its chemical composition. Its structure consists of stacked base-to-base columns of hollow polyhedral prismatic cells, filled with an air-like gas. The cells wall thickness varying between 1-1.5 µm in early cork to about 2 µm in late cork. [15]. The cellular structure of cork is made up of an internal primary wall rich in a thin layer of lignin, a thick secondary wall of alternating suberin and waxes and a tertiary layer of polysaccharides [15]. The relative abundance in which these components appear in the cork cells constitution is quite variable.

Several factors may influence the cork chemical composition, such as cork quality, geographical origin or morphological part of the tree from which cork was harvested [45]. There are also differences between virgin and reproducing cork [46]. Several studies were already reported concerning the chemical composition of cork. The average composition reported in the literature is summarised in Table 2.4. Suberin is among the most abundant component of cork. In fact, *Q. suber* is part of the restricted group of suberin rich species [16, 47], being this in the basis of several studies that have been done, exploiting this particularity of cork [48-50].

The cork cell walls are also constituted by the already mentioned non-structural components, not chemically linked, which can be easily removed by solvents [15, 16]. In general, the extractives content of virgin cork is higher than that of reproduction cork (Table 2.4).

Lipophilic and phenolic compounds, which can be extracted with non-polar and polar solvents, respectively, are the main components of cork extractives and the proportion in which they appear in cork composition is also variable [15, 16]. Castola and co-workers [51] reported an extraction yield of 6% for a dichloromethane extraction, while Sousa *et al.* [52] only achieved a value of 3.6%. Conde et al [53] also reported quite variable extraction yields (4.67-8.14%), between cork oak trees from different regions of Spain, using chloroform as solvent.

Extraction with polar solvents also showed to depend of the geographical origin of cork oak tree [54]. Extraction yield values ranging between 1 and 6.5% were achieved by using a mixture of methanol:water as extraction solvent [54, 55], while for the extractives soluble in ethanol and water were reported values of 4.8 and 4%, respectively [16].

The interest in the analysis of the phenolic fraction of cork is primarily associated to the potential migration of these compounds from cork stoppers to wine after bottling, with impact on its organoleptic properties [56-58]. Finally, the ash content of cork ranges between 0.5 and 4% and 0.7 and 5% for *amadia* grade and virgin cork, respectively (Table 2.4).

Table 2.4 – Summary of the chemical composition (%) of cork (adapted from [15])

Suberin	Lignin	Polysaccharides	Extractives	Ash	Source
Virgin cork					
45	21	13	19	1.2	[59]
38.6	21.7	18.2	15.3	0.7	[45]
35.2	22.4	21.3	16.9	0.9	[16]
45	27	12	10	5	[60]
Reproduction cork (*amadia* grade)					
50	19	13	15	3	[61, 62]
37	14.8		15.8		[63]
33.5	26	26	13	2.5	[59]
33	13	6	24		[46, 64]
42	21.5	16	13		[18]
62	23	21	11		[65]
39.4	24.0	19.9	14.2	1.2	[16]
48	29	12	8.5	2.1	[60]
33	33	23	9	4	[47]
41.3	13.4	7.4	19.8	0.5	[66]

2.2.3.1 Polysaccharides

After suberin and lignin, polysaccharides represent the most important component of the cork cell walls, conferring them structural rigidity [15]. Polysaccharides are composed mainly by cellulose, which represents about 51.9% of this fraction, and hemicelluloses [47]. The cork monosaccharides composition identified after hydrolysis consists mostly of glucose and xylose and lower amounts of arabinose, galactose, mannose and rhamnose (Table 2.5) [16]. Differences between virgin cork and *amadia* grade cork have been also detected. The last one has contents of arabinose and galactose higher than those of virgin cork, while the contents of the other monosaccharides are lower (Table 2.5).

Table 2.5 – Relative monosaccharides content (%) of virgin and *amadia* grade cork (adapted from [16])

Monosaccharides	**virgin cork**	***amadia* grade cork**
Glucose	50.7	45.4
Xylose	34.0	32.3
Arabinose	6.4	13.2
Galactose	3.6	5.1
Mannose	3.7	3.2
Rhamnose	1.7	0.8

Pinto and co-workers [47] also identified the monosaccharide composition of the non-cellulosic monosaccharides, identifying xylose as the main monomeric sugar, indicating the presence of xylans, followed by arabinose and 4-*O*-methylglucuronic acid. In fact, Asensio [67-69] identified three hemicelluloses in cork: a xylan, reported as hemicellulose A, composed by xylose with *β*(1-4) glycosidic linkages and 4-*O*-methylglucuronic acid in the position 2 of xylose [67]; another xylan (hemicellulose B-1) composed by xylose, 4-*O*-methylglucuronic acid, arabinose, galactose, mannose and glucose [68]; and a third hemicellulose (B-2) composed by xylose, arabinose, glucose, galactose, 4-*O*-methylglucuronic acid and rhamnose [69].

2.2.3.2 Lignin

Lignin, the second major component of cork cells, plays an important role on their structure, and, in fact, after its extraction the cells collapse [16]. This fraction of *Q. suber* cork is not fully understood. It has been reported that the lignin from cork as in *Q. suber* wood is of guaiacyl-type [16, 70, 71]. Several studies have been also carried out in order to understand the linkage between lignin and suberin [72, 73]. It was proposed that the aliphatic domain of suberin is separated from lignin, but interconnected by ester bonding with dicarboxylic and hydroxyfatty acids [15]. Furthermore, in cork, lignin also establishes linkage with hemicelluloses, resulting in lignin-carbohydrates complexes [16].

2.2.3.3 Suberin

The macromolecular composition of suberin, the main component of the cell walls of cork, is not completely established. However, it is known that suberin is an amorphous aliphatic-aromatic cross-linked polyester, being composed, mainly, by long chain aliphatic ω-hydroxyacids, α,ω-dicarboxylic acids, glycerol and phenolic compounds (Figure 2.11) [15, 48, 49]. It is impossible to remove suberin from cork cell walls without chemically degrading it.

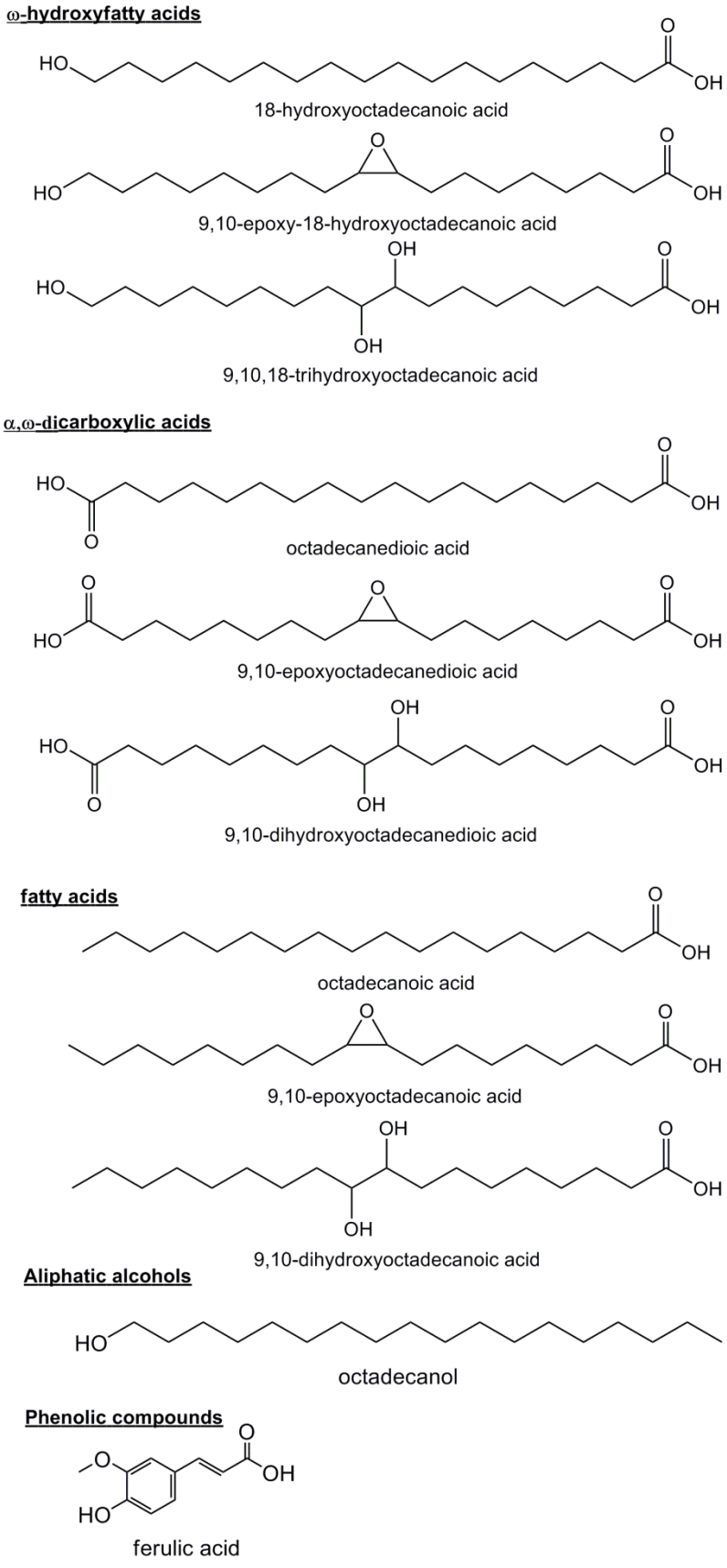


Figure 2.11 – Main monomeric components of suberin (adapted from [48])

Suberin depolymerisation has been carried out by alkaline hydrolysis or methanolysis [74-76], generating mostly ω-hydroxyfatty acids (26.3-61.7%), α,ω-dicarboxylic acids (6.1-53.3%), fatty acids (1.0-14.9%), with smaller amounts of long chain aliphatic alcohols (0.4-8.3%) and aromatic compounds (0.1-7.9%) [48]. Acids are characterised by the predominance of the C_{18} followed by C_{22} homologues and some of those can be functionalized in the middle of the chain by insaturations, vicinal di-hydroxy or epoxide groups (Figure 2.11) [48, 49]. Ferulic acid (Figure 2.11) is, in most of the cases, the compound detected in the aromatic fraction of cork suberin [74-76].

2.2.3.4 Inorganic components

The inorganic components of cork consist mainly by calcium (about 60%) and by lower contents of phosphorus, sodium, potassium and magnesium [16].

2.2.3.5 Extractives

Cork extractives can be divided in lipophilic (aliphatic and terpenic compounds) and phenolic compounds.

Aliphatic compounds in cork include *n*-alkanes, fatty acids and alcohols, glycerol and some triglycerides. The composition of this fraction of cork shows to be quite variable. Bescansa-Lopéz and Ribas-Marqués [77] reported the existence of about 0.6% of *n*-alkanes (C_{16}-C_{34}) in cork. Aliphatic alcohols represent about 1% of cork and comprise, mostly, the even members between C_{18} and C_{26} and in some cases with unsaturated groups (C_{20} and C_{21}).

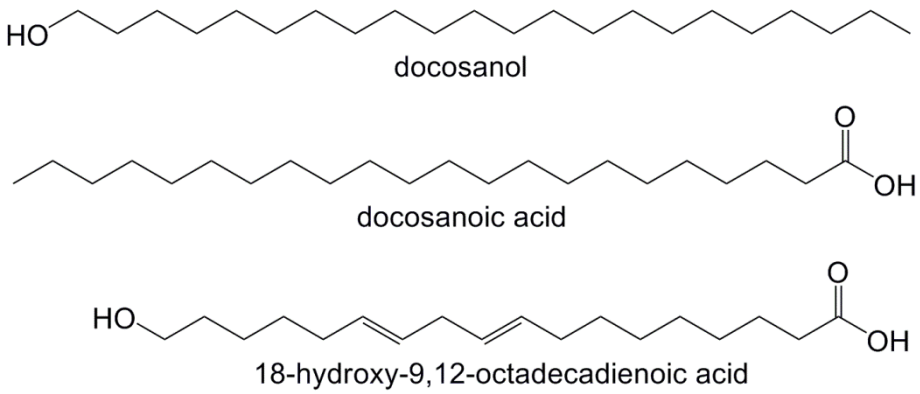


Figure 2.12 – Major aliphatic compounds in *Q. suber* cork

Both saturated and unsaturated fatty acids were identified in chloroform extracts of cork, all of them are even members between C_{16} and C_{24} [53, 78]. Some ω-hydroxyacids were also identified in cork extractives. Fatty acids represent about 0.85% of cork [78].

Docosanol, docosanoic acid and 18-hydroxy-9,12-octadecadienoic acids (Figure 2.12) are the main compounds from, respectively, the aliphatic alcohols, fatty acids and ω-hydroxyacids families present in cork [53, 77, 78].

About 50% of the lipophilic components in cork correspond to triterpenic compounds [15]. This fraction of cork extractives is composed mainly by friedelin, cerin, betulin and betulinic acid (Figure 2.13). Sousa *et al.* [52] reported that cerin is the major triterpenic compound in dichloromethane extracts from cork, followed by betulinic acid and friedelin. Castola and co-workers [51] have reported that friedelin can represent about 21% of a cork dichloromethane extract.

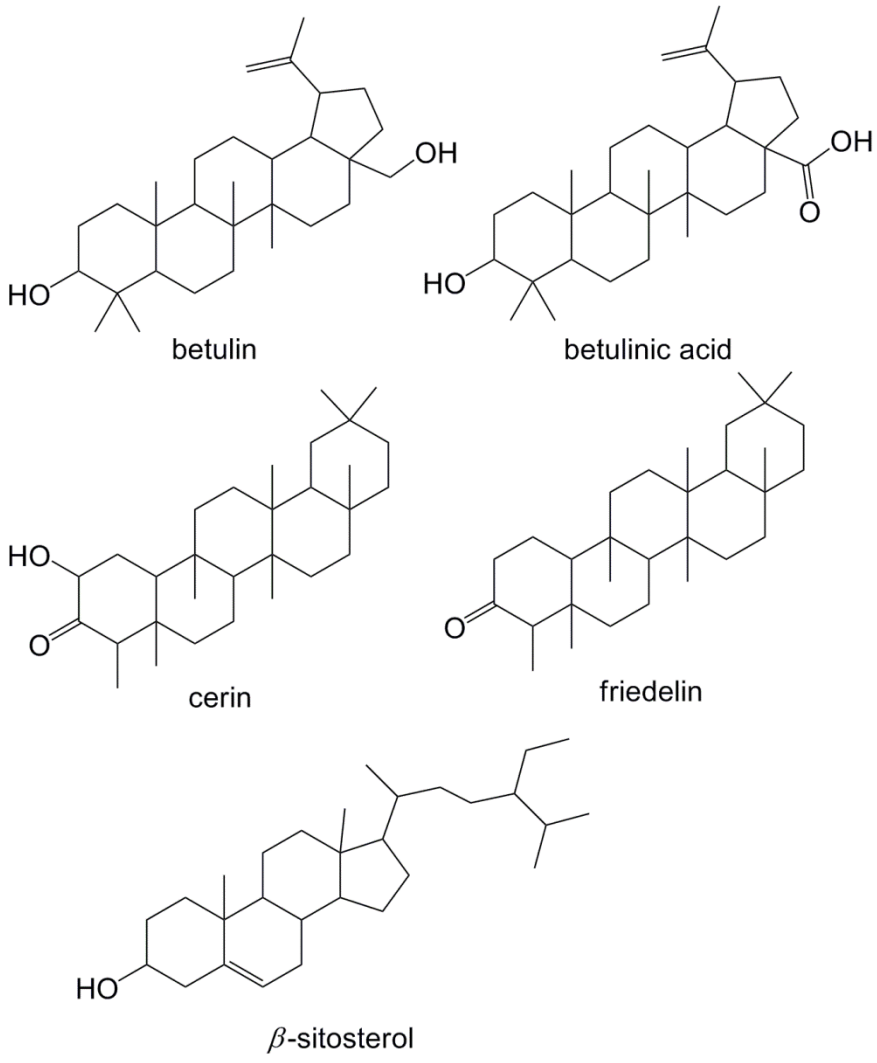


Figure 2.13 – Major triterpenic compounds in *Q. suber* cork

The lipophilic fraction of cork extractives contains also lower amounts of sterols, such as *β*-sitosterol (Figure 2.13) and other friedelane and lupane type coumpounds [51, 52].

Different phenolic compounds have been identified in *Q. suber* L. cork (Table 2.6). These compounds are commonly extracted from cork with methanol:water mixtures [54, 55, 79] or with wine model solutions [58, 80].

Phenolic acids have been reported as the main components of phenolic fraction of cork, in which ellagic, protocatechuic and vanillic acids (Figure 2.14) are among the major components. In fact, cork is particularly rich in ellagic acid (Figure 2.14), with contents ranging between 111 and 327 μg g^{-1} of dry cork [54]. High contents of ellagitannins, in particular of castalagin (Figure 2.14), were also reported [54, 79].

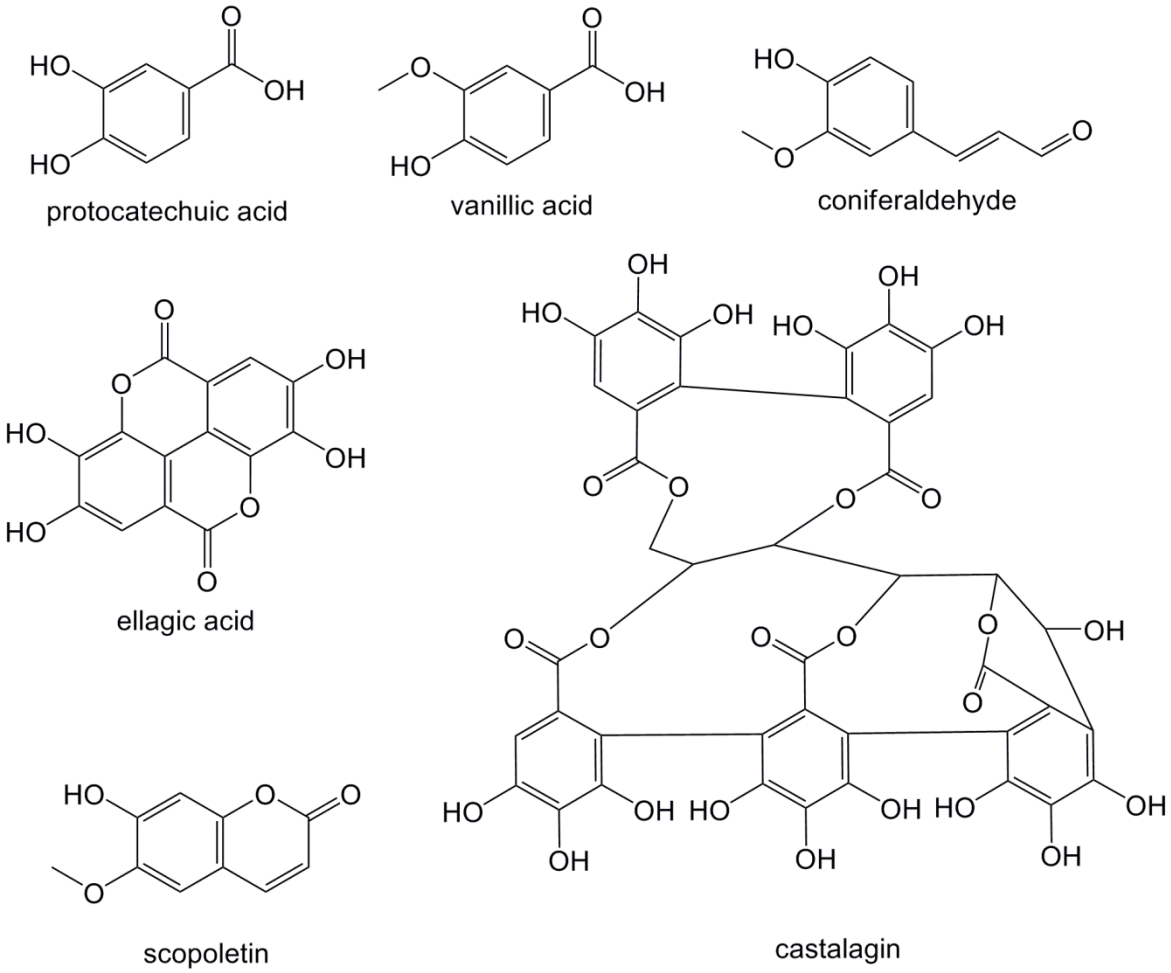


Figure 2.14 – Main phenolic compounds identified in *Q. suber* L. cork extractives

Several other phenolic oligomeric structures were also identified in cork from *Q. suber*, with a wide variety of gallic and ellagic acid derivatives [80]. It is estimated that 90% of phenolic fraction of cork is composed by tannins [16].

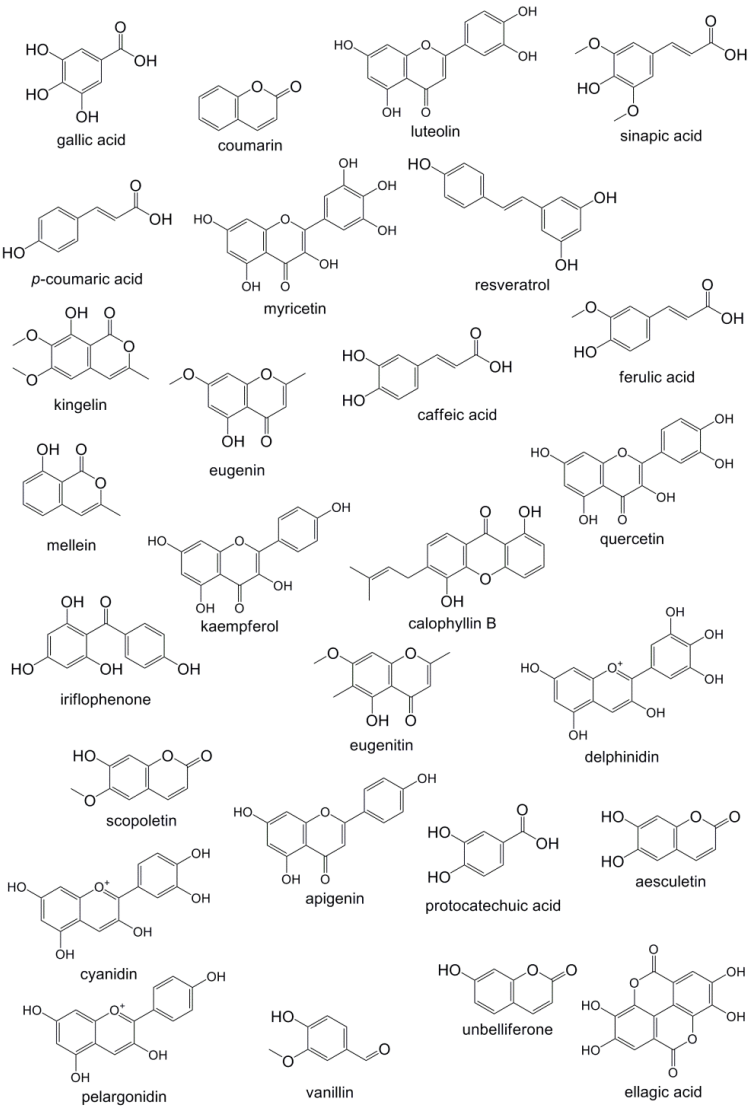
Table 2.6 – Phenolic compounds identified in *Q. suber* cork extractives

Phenolic compounds	Source
Benzoic acid	[58]
4-hydroxybenzoic acid	[58]
Protocatechuic aldehyde	[54, 55, 80, 81]
Vanillin	[54, 55, 58, 80, 81]
Protocatechuic acid	[54, 55, 80, 81]
p-coumaric acid	[58]
Acetovanillone	[58]
Gallic acid	[54, 55, 80, 81]
Esculetin	[54, 55, 81]
Coniferaldehyde	[54, 55, 80, 81]
Vanillic acid	[54, 55, 81]
Caffeic acid	[54, 55, 58, 80, 81]
Syringaldehyde	[58, 81]
Scopoletin	[54, 55, 81]
Ferulic acid	[54, 55, 58, 80, 81]
Sinapaldehyde	[54, 55, 81]
Ellagic acid	[54, 55, 80, 81]
Ellagic acid-pentoside	[80]
Ellagic acid-deoxyhexoside	[80]
Ellagic acid-hexoside	[80]
Valoneic acid dilactone	[80]
Hexahydroxydiphenoyl(HHDP)-glucose	[80]
Valoneic acid	[80]
Dehydrated tergallic-*C*-glucoside	[80]
HHDP-galloyl-glucose	[80]
Trigalloyl-glucose	[80]
di-HHDP-glucose	[80]
HHDP-digalloyl-glucose	[80]
Tetragalloyl-glucose	[80]
Vescalagin	[79, 80]
Castalagin	[79, 80]
Di-HHDP-galloyl-glucose	[80]
Trigalloyl-HHDP-glucose	[80]
Pentagalloyl-glucose	[80]
Grandinin	[79]
Monogolicain A/B	[80]
Roburin A/E	[79]

Lower amounts of cinnamic aldehydes, such as coniferaldehyde (Figure 2.14) and coumarins, such as scopoletin (Figure 2.14) [54, 55] have been also found in cork extracts. The relative abundance of each component shows to be variable with the geographic origin of cork oak tree [54].

Part B

Phenolic compounds, extraction and analysis



2.3 Phenolic compounds

Phenolic compounds constitute one of the most numerous and widespread group of plant secondary metabolites [82]. Thousands of phenolic compounds have been identified in edible and non-edible vegetal sources. These secondary metabolites are important to normal plant growth and development, and provide a defence mechanism against infection and injury [24]. The presence of phenolic compounds also may have an important function on the oxidative stability and microbial safety of injured plants [83]. Furthermore, these compounds are part of both animal and human diet with recognized beneficial effects at nutritional and health levels [84].

2.3.1 Phenolic compounds structures and classification

Phenolic compounds have as a common molecular base, a hydroxylated benzene ring (phenol). The structure of these compounds can vary between simple phenolic molecules up to complex polymeric compounds, with molecular weights higher than 30000 Da [84]. These compounds can be classified into different subgroups, depending on the functional groups attached to the phenolic unit [85]. A classification based on the number of carbons on the molecule was adopted by Harborne and Simmonds [86] and is presented in Table 2.7.

After lignin, which is the second most abundant biopolymer on Earth [85], phenolic acids and aldehydes, cinnamic acids, coumarins and chromones, benzophenones, xanthones and stilbenes, flavonoids and tannins are the more common phenolic compounds found in vegetal sources and will be focused in this bibliographic review:

Table 2.7 – **Classification of phenolic compounds (adapted from [86])**

Structure	Class
C_6	simple phenolic compounds
C_6–C_1	phenolic acids and related compounds
C_6–C_2	acetophenones and phenylacetic acids
C_6–C_3	cinnamic acids, cinnamyl aldehydes, cinnamyl alcohols
C_6–C_3	coumarins, isocoumarins and chromones
C_{15}	flavonoids
C_{30}	biflavonyls
C_6–C_1–C_6, C_6–C_2–C_6	benzophenones, xanthones and stilbenes
C_6, C_{10}, C_{14}	quinones
C_{18}	betacyanins
Lignans, neolignans	dimer or oligomers
Lignin	polymers
Tannins	oligomers or polymers
Phlopaphenes	polymers

2.3.1.1 Phenolic acids and aldehydes

These compounds are characterised by having, respectively, a carboxylic or an aldehyde group substituted on a phenolic structure. Gallic, protocatechuic and vanillic acids and vanillin (Figure 2.15) are common examples of this class of compounds [84].

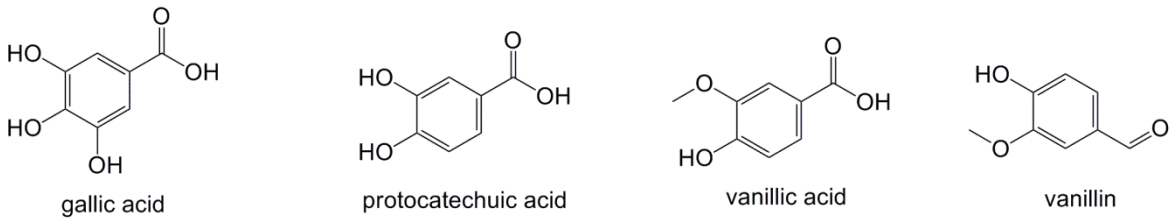


Figure 2.15 – Structures of some phenolic acids and aldehydes

2.3.1.2 Cinnamic acids

Cinnamic acids have a C_6–C_3 skeleton, being *p*-coumaric, caffeic, ferulic and sinapic acids the most abundant cinnamic acids found in plants (Figure 2.16). This class of compounds is also commonly found as esters of quinic or shikimic acids, as in the case of chlorogenic acid. Cinnamic acids are present in almost all plants, being dispersed through them in the seeds, leaves, stem and roots [87, 88].

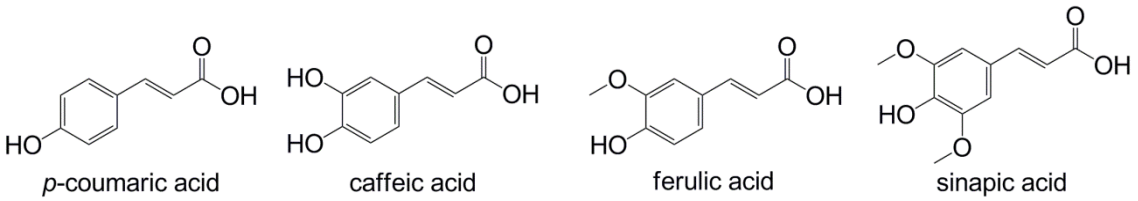


Figure 2.16 – Structures of some cinnamic acids

2.3.1.3 Coumarins, isocoumarins and chromones

Coumarins, also C_6–C_3 structures, are products of the cyclization of cinnamic acids, having an oxygen heterocycle in the C_3 unit. Cyclization of *o*-coumaric, *p*-coumaric, caffeic, and ferulic acids will result in coumarin, umbelliferone, esculetin, and scopoletin, respectively (Figure 2.17). It is estimated that more than 1000 coumarins have been identified from natural sources [89].

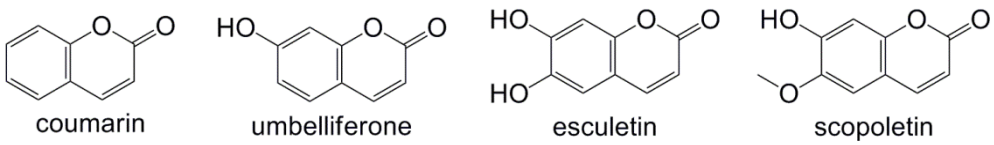


Figure 2.17 – Structures of some coumarins

Isocoumarins (Figure 2.18) are similar to coumarins, but the position of the oxygen and carbonyl groups are inverted [85].

Chromones also have a C_6–C_3 structure with a benzoannelated γ-pyrone ring (Figure 2.18). There is a large number of chromones and their derivatives identified in natural sources [90]. Flavones and isoflavones (described below) are 2- and 3-phenyl chromones, respectively.

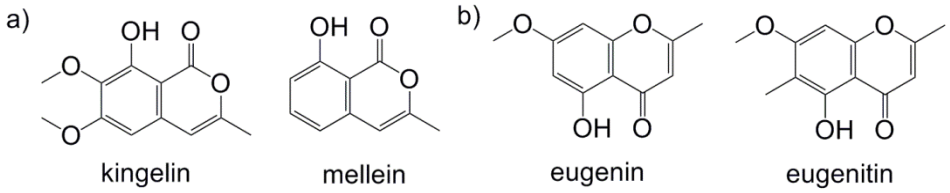


Figure 2.18 – Structures of some a) isocoumarins and b) chromones

2.3.1.4 Benzophenones, xanthones and stilbenes

Benzophenones and xanthones present a C_6–C_1–C_6 structure. The intramolecular cyclization of benzophenones generates xanthones. Stilbenes have a C_6–C_2–C_6 skeleton. The role of stilbenes in plants is related to its anti-fungal activity and protection against UV-induced oxidative stress. Furthermore stilbenes are found in wood and bark of several nematode-resistant species due, in particular, to its nematicidal activity [91]. Iriflophenone, calophyllin B and resveratrol are common examples of benzophenones, xanthones and stilbenes from natural sources, respectively (Figure 2.19).

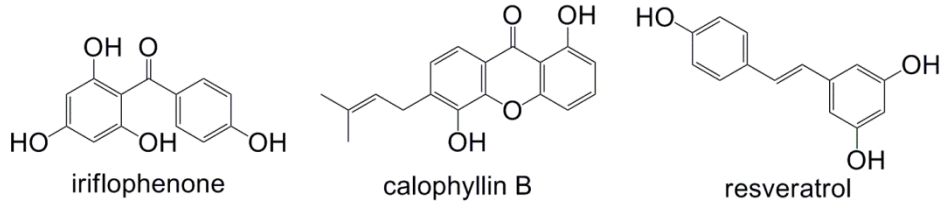


Figure 2.19 –Structures of, from left to right, iriflophenone, calophyllin B and resveratrol

2.3.1.5 Flavonoids

Flavonoids comprise the largest class of phenolic compounds, which have a 15-carbon (C_6–C_3–C_6) diphenylpropane skeleton. The 15-carbon backbone consists of two aromatic rings, ring A and ring B, which are attached by a three carbon chain (C_3). Differentiation between flavonoids is based on the structure of the C_3 group and its level of oxidation [92]. If the C_3 group is part of a pyranic heterocycle (C-ring), the phenolic classes will be classified as flavones, isoflavones, flavonols, flavan-3-ols, flavan-3,4-diols, flavanones, and anthocyanidins (Figure 2.20).

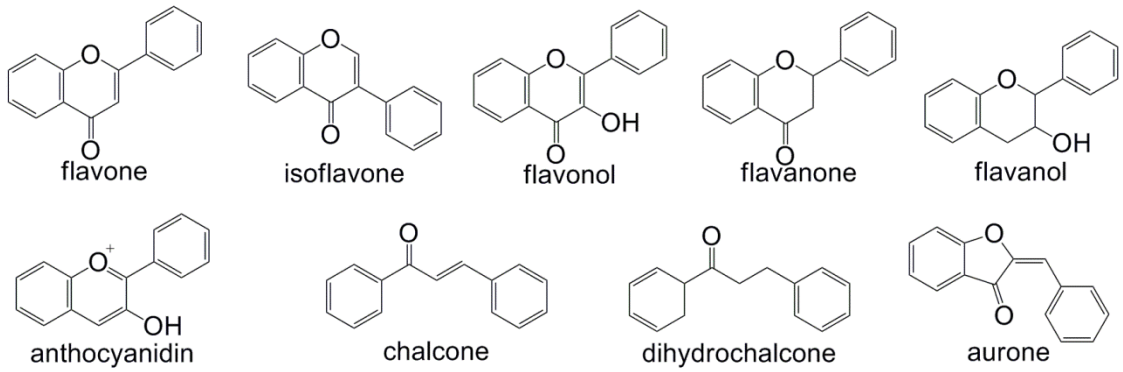


Figure 2.20 – Generic structure of the main flavonoid classes

Chalcones and dihydrochalcones (Figure 2.20) represent the second largest group of flavonoids. These compounds have an open C_3 chain linking the two aromatic rings, with a double bond in the case of chalcones and saturated in the case of dihydrochalcones. Aurones, which are products of the cyclization of chalcones, have a five-membered ring instead of the more common flavonoids [92].

Despite the large variety of aglycones present in plants, kaempferol, quercetin, myricetin, apigenin, luteolin and anthocyanidins (Figure 2.21) are among the most common and frequently appear connected with sugar moieties [92].

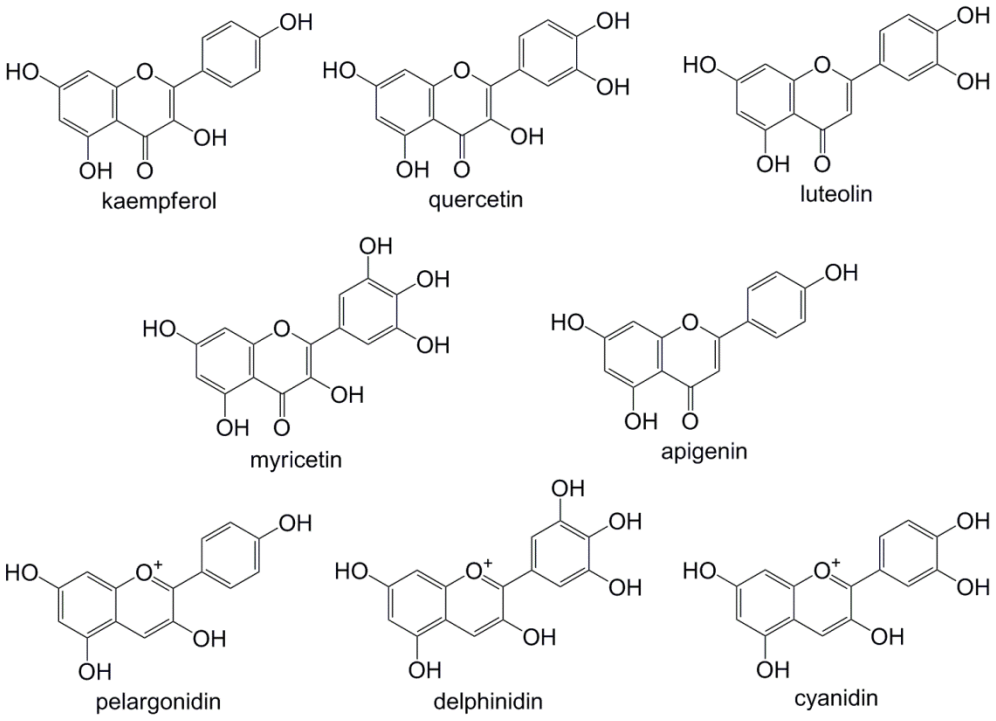


Figure 2.21 – Structures of the main flavonoid aglycones

Flavonoids also occur in vegetal sources in a combined form as glycosides, which can contain either *O*- or *C*- bonds. The sugar units vary between glucose, galactose, xylose, arabinose and rhamnose. The function of flavonoids and their glycosides in plants is related to the protection against UV-B radiation and against microbial invasion [93]. Anthocyanins (glycosides of anthocyanidins) are also responsible to the colour of flowers and fruits [92, 93]. The colour can range from yellow to purple, depending of several factors, such as the pH, the type of aglycone or even the extent of glycosilation [92].

2.3.1.6 Tannins

Tannins are phenolic compounds characteristic of higher plants and can be found in the bark, fruits or leaves of those vegetal sources. These compounds protect the plant against infection, insects or herbivory [94, 95]. Tannins include phenolic compounds with a wide structural diversity and can be classified as condensed, hydrolysable and complex tannins [96]. This classification is represented in the scheme of Figure 2.22.

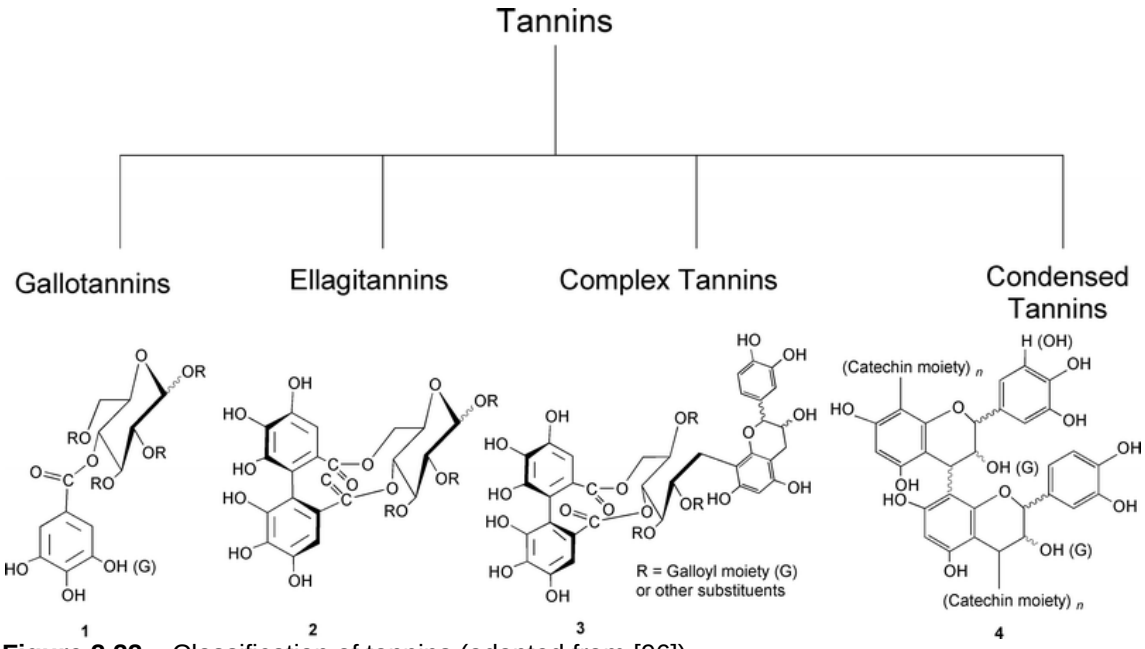


Figure 2.22 – Classification of tannins (adapted from [96])

Condensed tannins, also known as proanthocyanidins, are polymers of flavan-3-ol (catechin) units linked by C-C bonds. These compounds can have a galloyl moiety linked to the hydroxyl group at C-3 of each catechin unit (Figure 2.22) [96]. These compounds are well known components for example of red wine [94, 96], being astringency one of their main characteristics, due to their ability to complex and precipitate proteins [94, 96, 97]. This capacity increases with the degree of polymerization [96].

Hydrolysable tannins are divided into gallotannins and ellagitannins. Gallotannins have a carbohydrate core, mostly glucose, substituted with up to 12 gallic acid residues, bond to the core through ester functionalities. Furthermore, the gallic acid residues can be attached between them by *meta*- or *para*-depside bonds. [85, 94]

Ellagitannins are derived from pentagalloylglucose, with additional C-C bonds between adjacent galloyl moieties, which forms a hexahydroxydiphenoyl (HHDP) unit [96].

Complex tannins are compounds in which a catechin molecule is connected with gallotannins or ellagitannins units by a glycosidic bond [96].

2.3.2 Phenolic compounds properties

The properties assigned to phenolic compounds are almost endless and, in recent years, there was a large increase on the search of the health benefits of these compounds [98, 99]. One of the main factors which leaded to the large interest on the characterisation of phenolic compounds from natural sources and evaluation of their health beneficial effects was the search for the phenolic compounds responsible for the well known dietary and medicinal properties of some plant species [100]. In addition, these studies were encouraged by the development of powerful separation and characterisation technologies in the last decade. Actually, the evolution of publications about the phenolic compounds properties along the last decade increased more than six times (Figure 2.23).

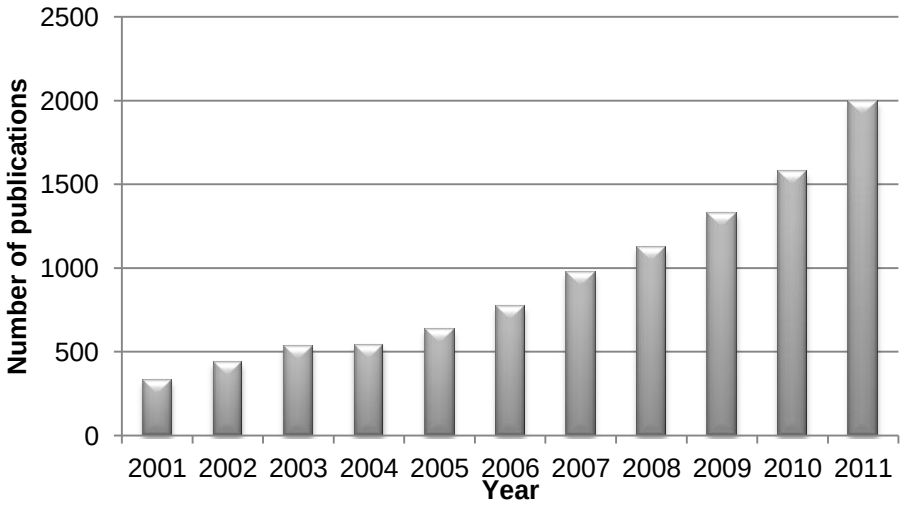


Figure 2.23 – Evolution of the number of publications in the last decade concerning the keywords in topics search:“phenolic compounds” and “activity” (ISI – Web of knowledge, 2012 October)

Despite the numerous chemical, biological and pharmacological properties assigned to phenolic compounds their most explored property is the antioxidant activity [101-105].

Additionally, many studies have reported the positive effect of phenolic compounds against some currently important diseases. Hassan Khan and Ather [106] showed that several phenolic compounds exhibit inhibitory effects against several steps of HIV-1 life cycle. Gallic acid has shown promising properties as antimelanogenic agent [107]. Excellent inhibitory effects for epigallocatechin-3-gallate and curcumin in the amyloid fribril formation have been found [108]. Zhang *et al.* [109] reported the inhibition of the growing human oral, colon and prostate cancer cells by some phenolic compounds isolated from strawberry extracts, including ellagic acid and anthocyanins. Some authors also have reported the promise of tannins as being used on the treatment of non-insulin dependent diabetes mellitus, due to their capacity to enhance glucose uptake and inhibit adipogenesis [110]. Table 2.8 summarizes some of the main health benefits reported for phenolic compounds, according to their classes.

Table 2.8 – Summary of some of the health benefits of the different classes of phenolic compounds

Group	Activities	Source
Phenolic acids and related compounds	Antioxidant	[103]
	Antimelanogenic	[107]
	Antiproliferative	[111]
	Antimicrobial	[112]
Cinnamic acids	Antioxidant	[113]
	Anti tuberculosis; Antidiabetic; Antioxidant;Antimicrobial; Hepatoprotective; Anticholesterolemic; Antifungal; fungitoxic; Antihyperglycemic; Antimalerial; Antiviral; Anxiolytic; Anti-inflammatory; UV rays absorbent	[114]
Coumarins	Antioxidant; Antithrombotic	[89]
	Antibacterial; spasmolytic	[115]
Chromones	Anti-inflammatory	[116]
	Anti-HIV	[117]
	Antioxidant; Anti-inflammatory; Anti-tumor; Antiviral; Antimycrobial; Enzyme inhibition; Antifeedant activity; Diuretic activity	[90]
Benzophenones	Antimicrobial; antioxidant; anti-inflammatory	[118]
Xanthones	Antioxidant; Anti-inflammatory; Platelet aggregation inhibition; Antithrombotic; Vasorelaxant effect;	[119]
Stilbenes	Antioxidant; Anti-cancer on mammary, prostate, colon and colorectal carcinomas and on childhood lymphoblastic leukemia; Cardioprotective; Anti-inflammatory; Anti-fungal; Anti-alergic; Anti-diabetic	[120]
Flavonoids	Antioxidant; Inhibition of enzymes; Anti-inflammatory; Cytotoxic antitumor; Oestrogenic; Vascular; spasmolytic; Hepatoprotective; Antibacterial; Analgesic; Malaria prevention	[93]
	Antioxidant; Antithrombotic; Total cholesterol decreaser; Antimutagenic; Antiangiogenic	[121]
	Anti-inflammatory; Antioxidant; Antiallergic; Hepatoprotective; Antithrombotic; Antiviral; anticarcinogenic	[122]

Group	Activities	Source
Hydrolysable tannins	Antimicrobial	[123]
	Antioxidant; Antimutagenic; Anti-tumor promotion on skin and gastrointestinal tract tumors; Antiviral; Anti-HIV activity; Antibacterial	[124]
Condensed tannins	Antimicrobial	[123]
	Antioxidant; Antimutagenic; Anti-tumor promotion on skin and gastrointestinal tract tumors	[124]
	Anti-angiogenic against lung, breast, colon and prostatic cancer	[125]

Extracts rich in phenolic compounds have been used worldwide in nutraceutical, pharmaceutical and cosmetic fields [126]. However, in the last decade, the extracts have been substituted by the active phenolic compounds. As example, ferulic acid has been used as food preservative or as ergogenic component in sport drinks [127], coumarin derivatives have been used as anti-thrombotic agents [128] and derivatives of resveratrol has also been used as components of skin creams [129].

Despite the exploitation of phenolic compounds is mainly related to their beneficial health effects, these compounds are used worldwide in industry for different purposes. The already described ability of condensed tannins to complex proteins has been explored, since the ancient times, to convert raw animal hides into leathers [96]. These compounds also have the capacity to complex with metal ions and polysaccharides [96, 97]. Furthermore several tannins are used worldwide to clarify beer, juice or wine or in the rubber production as anticoagulant [96].

2.4 Phenolic compounds extraction

The extraction of phenolic compounds from vegetal sources is a complex task, due to the dependence of several parameters as the sample particle size, the extraction method employed, the extraction time, the storage conditions and the presence of interfering components [130]. Furthermore, the extraction conditions must be carefully chosen in order to avoid possible degradation of phenolic compounds. Temperature, air and light are the main factors that promote the degradation reactions of phenolic compounds [131, 132]. The thermal degradation of these compounds is described as an oxidative process, which requires the presence of oxygen [132]. The presence of light is reported to catalyze the reaction of transformation of biological active forms (isomers) of phenolic compounds into inactive forms [133]. It has also been reported that longer extraction times increases the possibility of enzymatic degradation [131].

Due to the vast range of phenolic compounds and their presence in natural sources vary infinitively, there are no optimal extraction conditions to be applied to all the plant sources, however, there are several considerations that must be applied in most of the cases. One of the main is the temperature used in the drying and extraction processes, which could lead to chemical and enzymatic degradation or losses by volatilization or thermal decomposition of the phenolic compounds [134]. There is also consensus that lower particle size favours the extraction of phenolic compounds [135, 136].

The choice of the extraction solvent or solvent mixtures is one of the main variables to pay attention, and, from which, the success of the extraction process depends drastically. Organic solvents are still the most successfully applied. Methanol, ethanol, acetone, ethyl acetate and diethyl ether are the most common solvents chosen for the extraction processes. Several authors described satisfactory results when binary or ternary mixtures between those solvents are used [137-139] There is also a number of studies using these solvents acidified [137, 140]. In fact, the extraction pH is a variable which affect considerably the process, particularly when it is intended to extract not only the free phenolic compounds but also the insoluble-bound ones, such as those linked to lignin [130, 134]. Acidic and alkaline hydrolysis prior to extraction are commonly applied to release wall-bound phenolic compounds, as esterified phenolic acids or to remove the sugar moieties from flavonoid glycosides [130, 134, 141, 142]. Enzymatic hydrolysis is also used to release phenolic compounds [134, 142].

Sequential extraction steps must be considered if non-phenolic compounds, such as waxes, terpenes, fats and chlorophylls, are to be previously removed from the vegetal matrix.

Solid-liquid extraction is still the most common procedure to analyse the phenolic fraction of plant sources, due to its simplicity, high efficiency and easy tuner. However, over the last decades more efficient and/or environmentally friendly extraction methods have been studied, such as accelerated solvent extraction, ultrasound-assisted extraction, microwave-assisted extraction and supercritical fluid extraction. There is already a large interest in use environmentally friendly solvents. Ionic-liquids, for example, known as green and designer solvents, have been applied in several extraction techniques [143].

2.4.1 Conventional solid-liquid extraction

Conventional solid-liquid extraction, also called maceration, in which the compounds are removed from the vegetal matrix by submerging it in water, an organic solvent or solvent mixtures, is a traditional extraction technique used to extract phenolic compounds [142, 144]. This procedure can be performed at room temperature or at high temperatures, with a reflux apparatus. Apart from the solvent and temperature used, the main parameters affecting this technique are the solid/volume ratio and extraction time [145-147]. In some studies, mechanical means to increase molecular interaction are employed, such as vortex followed by centrifugation or mechanical stirring [139, 146].

This method was chosen for several authors for the extraction of phenolic compounds from *Eucalyptus* barks including *E. globulus* [38, 40, 43]. Methanol:water mixtures in a proportion of 80:20 were the most frequently selected solvent mixture used to extract phenolic compounds from *Eucalyptus* bark [38, 41, 43]. Furthermore, most of the studies also carried out the extractions at the room temperature for 24 hours and used a solid/liquid ratio of 1/60 (m/v) [38, 41, 43]. Recently, Vázquez *et al.* [29] compared the phenolic extracts of *E. globulus* bark obtained by conventional solid-liquid extraction using different solvents and mixtures, with methanol:water in a proportion of 50:50 showing better results: the solid/liquid ratio used in this study was 1/10 (w/w) and the extraction time varied between 1 and 2 hours [29]. Nevertheless, this study was done applying high temperatures (near or at the boiling point of solvents).

Conventional solid-liquid extraction has been also applied in the extraction of phenolic compounds from cork. The solvent mixtures used vary between wine model solutions, with a solid/liquid ratio of about 1/17 (m/v), [80, 148], and methanol:water (80:20) mixtures, with a solid/liquid ratio of 1/75 (m/v), followed by liquid-liquid extraction with diethyl ether [54, 55]. Furthermore the extraction of phenolic compounds from cork in these studies was always at the room temperature and the extraction time varied between 24 [55] and 72 hours [80, 148].

Due to its simplicity, easy adjustment, and efficiency, this technique could be considered the appropriate choice in a first approach to evaluate the phenolic composition of plant sources. However, due to its time-consuming, large consumption of organic solvents (often toxic) and, consequently, high costs, in an industrial application perspective more environmentally friendly and sustainable techniques must be considered.

2.4.2 Soxhlet extraction

Soxhlet extraction is an alternative to conventional solid-liquid extraction technique, that can be use to extract phenolic compounds from solid matrixes. This method involves the continuous extraction of a solid sample with a limited amount of solvent, due to the sequential steps of distillation/condensation of the solvent over the sample. Despite its simplicity and inexpensive extraction apparatus (Figure 2.24), this technique requires long extraction times and extraction temperatures at the boiling point of the solvent used, which in some cases could not be compatible with thermo-labile compounds [150].

In fact, the temperature required to boil the solvent, can be a clear disadvantage of this methodology. Although some authors have reported higher extraction yields and phenolic compounds contents by using soxhlet extraction instead of other techniques [149, 151], in most cases soxhlet extraction shows to be less efficient. Annegowda *et al.* [152] extracted phenolic compounds from *Bauhinia purpurea* L. leafs by a soxhlet extraction for 48 hours with ethanol, and by a conventional solid-liquid extraction, also with ethanol, but at room temperature and for 3 days. Despite the extraction yield obtained by soxhlet extraction was slightly higher, the phenolic compounds content, as well as the content of flavonoids and tannins, were significantly lower [152]. Budrat *et al.* [153] also reported that the phenolic compounds content of extracts from bitter melon obtained by soxhlet extraction, using methanol as solvent for 4 hours, is clearly lower than those obtained by other extraction techniques.

Tomsone and co-workers [149] compared several solvents and solvent mixtures in a soxhlet extraction of phenolic compounds from horseradish roots and verified that 95 % ethanol is the most efficient. Recently, Vazquéz *et al.* [29] also applied soxhlet extraction during 15 hours with different organic solvents to compare the phenolic compounds content and antioxidant activity of *E: globulus* bark extracts. However, this technique is most commonly applied in the extraction of lipophilic fraction of natural matrices. With non-polar solvents, such as n-hexane or dichloromethane, the undesired compounds can be firstly extracted from the plant matrix. In fact, several studies have successfully used soxhlet extraction with dichloromethane to extract the lipophilic fraction from *Eucalyptus* bark [31, 32, 154] and from cork and cork by-products [52], applying extraction times of 7 and 10 hours, respectively.

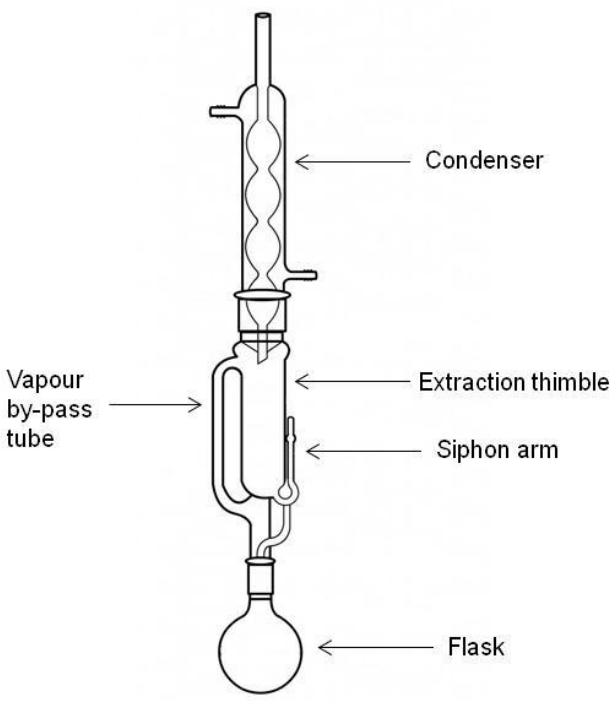


Figure 2.24 – Scheme of a soxhlet extraction apparatus

2.4.3 Accelerated solvent extraction

Accelerated solvent extraction (ASE), also known as pressurized liquid extraction (PLE), has become another promising extraction process for phenolic compounds, providing lower extraction time and solvents consumption. This technique uses organic solvents at high pressure and temperature (above their boiling point), in order to accelerate the extraction process [155, 156]. On the one hand, the high temperature increases the extraction kinetics, as at the same time, by decreasing the viscosity, it enhances the sample penetration. On the other hand, the high pressure allows the solvent to stay at liquid state and facilitates the desorption of analytes [155]. One of the main advantages of this technique is that, although the high temperatures used, ASE does not causes degradation of phenolic compounds, due to the absence of air and light during this extraction process [132]. This fast and automated technique provides successful results in the extraction yield and efficiency of extraction. Furthermore, the recent developments in ASE technology allow the use of green solvents, as water or even ethanol, which is generally recognized as safe [156].

2.4.4 Ultrasound-assisted extraction

Ultrasound-assisted extraction (USAE), in which the solid matrix is immersed in a solvent and submitted to ultrasound irradiation (US), by using an US bath or probe, has become an emerging technique in the extraction of phenolic compounds. It uses sound waves at frequencies over the audible to humans value (20 kHz) to disrupt the plant cell walls, thus, accelerating the solvent penetration into the plant material and enhancing the release of the target analytes [157, 158]. Therefore, USAE requires lower extraction times than maceration, soxhlet or even microwave-assisted extraction (MAE). Furthermore, this technique involves lower temperatures, which is an advantage in the case of temperature sensitive compounds, such as the phenolic compounds [157]. USAE has been well accepted by food industry, being already applied, not only in the extraction of natural compounds, as also in processing and preservation operations [158]. Notwithstanding, USAE, when compared with supercritical fluid extraction (SFE), is described as less selective, efficient and with higher extraction times, although the USAE equipments are simpler and less expensive than SFE apparatus [158, 159].

2.4.5 Microwave-assisted extraction

Microwave-assisted extraction (MAE) is an efficient technique for phenolic compounds extraction, which is applicable to thermally stable compounds, with the advantages of its short extraction times and lower solvent volumes. Microwave energy is a non-ionizing radiation that causes molecular motion, mainly by dipolar rotation [160]. The optimisation of MAE process involves several parameters, such as, temperature, type of solvent and its volume, irradiation time and power. Binary mixtures with acetone, methanol or ethyl acetate and water have been commonly applied in MAE extraction of phenolic compounds [138]. The efficiency of MAE is dependent on the dipole moment for microwave energy absorption of the solvent chosen [160], as well as in the nature of the solid matrix. The existence of some water content in solid matrixes promotes the release of extractives into the surrounding medium [160]. Although MAE is carried out in closed systems, without air and light, the temperature is, once more, an important variable, concerning the stability of phenolic compounds. Liazid and co-workers [131] have demonstrated the degradation of some phenolic compounds when submitted at temperatures above 100 ºC in MAE process using methanol as solvent. The establishment of new analytical methods concerning environmental issues has become object of study. In fact, some prospective results have been already obtained by using ionic-liquids [161] or even ethanol and water [162] in the MAE extraction of phenolic compounds from vegetal sources.

2.4.6 Supercritical fluid extraction

Supercritical fluid extraction (SFE) is becoming an attractive alternative method for conventional solid-liquid extraction of organic compounds from biomass materials [163, 164]. A supercritical fluid is a substance that, at temperatures and pressures higher than its critical temperature and pressure (the critical point) (Figure 2.25), shows compressibility, transportation and penetration properties of a gas and the densities and solvating power of a liquid [163]. Furthermore, in comparison to common solvents, supercritical fluids exhibit higher diffusivities, lower viscosities, almost null surface tensions, and similar densities, which combined provide them excellent solvent and operational characteristics [165]. Moreover, their properties can be tuned by changing the temperature and pressure, or through the addition of a co-solvent.

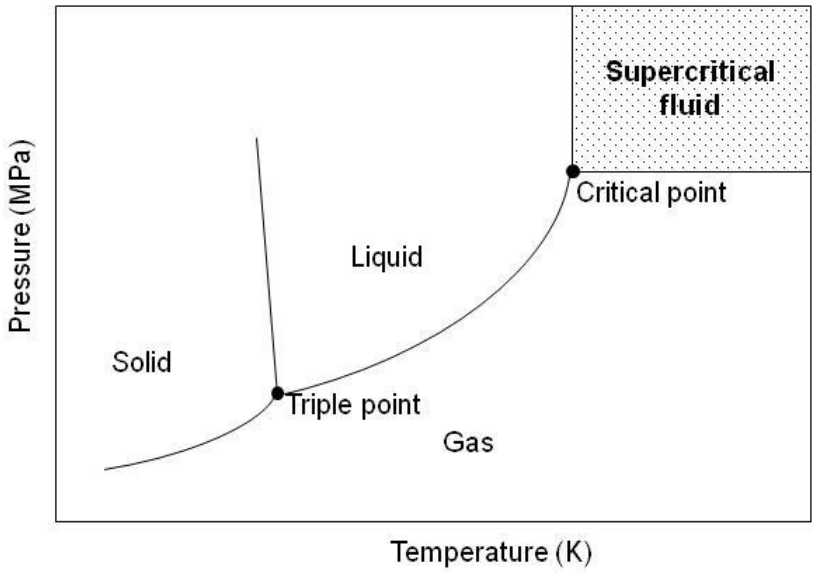


Figure 2.25 – Schematic pressure-temperature phase diagram of a pure substance

Carbon dioxide (Pc = 7.28 MPa, Tc = 304.1 K [165]) is the most widely used fluid for SFE, since it is non-toxic, environmentally safe, non-flammable, low cost at high purity, and easily removed from final extracts. Additionally, it allows the use of relatively low pressures and near room temperature conditions, and the absence of light and air in the process reduces the possibility of oxidative degradation [166], which could be an acute problem in the case of phenolic compounds. Due to its non polarity, CO_2 is not an efficient solvent to extract polar compounds, such as phenolic compounds. Addition of modifiers, such as ethanol, methanol or even water, enhances the solvating power of CO_2, increasing the selectivity and therefore the yield of target compounds. Ethanol is the most used supercritical CO_2 modifier, because it is an environmentally friendly solvent, allowed

in food, cosmetic and pharmaceutical industry [165]. Furthermore, it can be easily eliminated from the extraction vessels by evaporation, and recycled to the system.

2.4.7 Solid phase extraction

Solid phase extraction (SPE) is an extraction technique commonly applied in liquid matrixes, such as juices [167], allowing at the same time to fractionate the extracts into groups of components. This technique can be also applied in solid matrixes, after their pre-extraction into solvents. Therefore, SPE allows the sequential fractionation of different classes of phenolic compounds and removal of unwanted components [130, 168]. This method is based on chromatographic processes, during which analytes are adsorbed in a solid sorbent and subsequently eluted by sequential elution with appropriate solvents. The optimisation of the SPE process involves the choice of the type of sorbent and solvent. This process can also be automated, being already available several commercial cartridges with a wide variety of sorbents. Non-polar reversed-phase silica based sorbents, mostly C_{18}, are the common used cartridges in phenolic compounds fractionation [130, 142].

2.5 Phenolic compounds analysis

The analysis of phenolic rich extracts is a complex task that has been facilitated in the last decade by the technological evolution of many spectroscopic techniques. Nevertheless, the characterisation of a complex material always requires the use of several techniques: starting from global gravimetric and spectrophotometric methods; passing by chromatographic techniques coupled to mass spectrometry; and ultimately by the fractionation of the extracts to obtain pure compounds for characterisation by more powerful spectroscopic techniques, such as NMR and X-ray analysis.

2.5.1 Spectrophotometric analysis

Diverse spectrophotometric methods have been used to estimate either the total phenolic content (TPC) or the content of a given class of phenolic compounds, such as hydrolysable or condensed tannins.

2.5.1.1 Total phenolic content

The most common used method to determine the TPC is the Folin-Denis method [169], modified by Folin and Ciocalteu [170], although some studies [171] have been also carried

out using the Prussian Blue assay [172]. These assays are based on oxidation-reduction reactions, in which the phenolate ions are oxidized and the $Fe(CN)_6^{3-}$ ion (in Prussian Blue assay), or phosphotungstic-phosphomolybdic (in Folin-Denis assay) are reduced, forming coloured products. As all the free hydroxyl groups could participate in the reaction, these assays just give an idea of the amount of total phenolic compounds present in the extract. These two methods were firstly developed to detect the aminoacid tyrosine in protein hydrolisates [173].

Some limitations have been attributed to Prussian Blue method, namely the formation of a precipitate and the increase of colour density along the time [97]. Furthermore, pH and temperature have been described to affect the colour density of the products [174].

Initially, Folin-Denis assay was also limited with the formation of a precipitate. However, a modification made by Folin and Ciocalteu [170], introducing lithium sulfate and bromine in the reagent mixture (Folin-Ciocalteu reagent), solved that problem. Later Singleton and Rossi [175] introduced some modifications to the conditions, in order to turn reduction reaction more specific for phenolic compounds and to reduce the variability of the results.

The chromophore phosphotungstic-phosphomolybdic complex, formed in Folin-Denis assay, presents a blue colour, which intensity could rapidly be quantified by absorbance reading in a UV-vis spectrophotometer at 760 nm. Although studies have been done using different phenolic compounds as reference standards [176-178], gallic acid, which was proposed by Singleton and Rossi [175], is the most commonly used reference [104, 105, 179], being the final results expressed as gallic acid equivalents.

In recent years a number of limitations have been attributed to this assay [97, 180, 181], mostly related to the less of sensitive of the method, with the contribution by non-phenolic compounds, which could lead to an overestimation of the results. However, the main problem related with this assay is the lack of a standardized methodology [180]. Several authors have been reporting different conditions in what concerns the volume, concentration of the solutions or incubation time [101, 104, 105, 179]. However, a large number of studies [140, 182-184] have used the conditions proposed by Scalbert *et al.* [185]. Moreover the major studies concerning the bark of several *Eucalyptus* species and the *Q. suber* cork have applied the same procedure [43, 55, 186, 187].

Folin-Ciocalteu method can continue to be a rapid approach in the total phenolic content, even to comparative purposes, considering the same methodology conditions and the same reference standard.

2.5.1.2 Hydrolysable tannins content

Several methods have been described to estimate the total amount of hydrolysable tannins [185]. The potassium iodate assay, firstly described by Haslam *et al.* [188], and later modified by Bate-Smith [189], is based on the reaction between potassium iodate with gallo- and ellagitannins, producing a pink reaction product. However, numerous problems are attributed to this test, as a high sensibility for oxygen content, temperature and reaction time, leading to an unstable coloured product [190]. These leaded some authors to suggest several modifications to the method [191, 192].

Another method used for the estimation of hydrolysable tannins, in particular gallotannins, is the rhodanine assay, developed by Inoue and Hagerman [193]. In this test, the reagent (2-thio-4-ketothiazolidine) reacts specifically with gallic acid, under anaerobic conditions, forming a red product. Gallotannins are determined, after a hydrolysis step, by the difference of gallic acid quantity before and after hydrolysis. Some criticisms have been done for this assay [190], attributing interference of gallic acid molecules presented in some ellagitannins, as well as due to the variation in the number of gallic acids moieties between the diverse naturally occurring gallotannins. Bate-Smith [189] described an analytical procedure to estimate only the ellagitannins, the sodium nitrite assay, later improved by Wilson and Hagerman [194]. This assay is based on the formation of a red chromophore during the reaction of ellagic acid units, resulted from the ellagitannins hydrolysis, with the electrophile NO^+. Despite some underestimation of this method has already been reported [195], the main advantage of this assay is that when other compounds, as gallic acid or hydroxycinnamic acids react with the electrophile the products formed have a yellow-brown colour, no interfering with the colorimetric test [181]. Nevertheless this assay has the counterpart of to be necessary a large amount of pyridine, used as solvent.

Despite some authors included these assays in their studies [196-198], the estimation of total hydrolysable tannins content is not a widely used procedure. Actually, no study concerning the characterisation of phenolic fractions of *Eucalyptus* bark or *Q. suber* cork has used these assays.

2.5.1.3 Condensed tannins content

Several colorimetric assays have been described to estimate the total condensed tannins content, with special emphasis in grains [130]. The vanillin test, which involves the reaction of condensed tannins with vanillin under acidic conditions, is probably the most

used. However, the sensitivity of this method to monomeric flavonols, as well to dihydrochalcones becomes a disadvantage [130, 199]. Furthermore, the success of this assay is dependent on the nature of the solvent, nature and concentration of the acid, temperature, reaction time and reference compounds used [97].

Other colorimetric assay relies on the conversion of proanthocyanidins in red anthocyanidins, in the presence of a solution with butanol and chloride acid. Due to the reproducibility of the method and the yield of conversion increases in the presence of transition metals, ferrous or ferric ions are strongly suggested to add to the reaction mixture [200]. However, the use of this method have to take into account several other considerations, due to its sensitivity to the degree of polymerization of proanthocyanidins, the number of hydroxyl groups in their rings, the ratio acid-butanol or even to the quantity of water in the reaction medium [97]. These assays are commonly applied in the study of seeds [201, 202], due to its well-known high content in proanthocyanidins, but several authors already used it in the study of other sources as *Q. suber* cork [54] or *Eucalyptus* bark [43]. However, the assay mostly used in woody species, in particular in the bark, is that proposed by Yazaki and Hills [203], which involves the precipitation of condensed tannins with formaldehyde. This reaction, originally described by Stiasny [204], is catalyzed by chloride acid and the precipitate obtained is weighed to obtain the yield of precipitate (Stiasny number). The Stiasny number has been determined also to evaluate the potential of extracts to wood adhesives applications [205]. Despite its time consuming, this assay has been used worldwide in the analysis of condensed tannins from the bark of numerous species [206, 207], inclusively of *E. globulus* [186, 187].

2.5.2 Chromatographic techniques

Chromatography has become a central technique for both separation and quantification of phenolic compounds. Gas chromatography (GC) and high-performance liquid chromatography (HPLC) have become the most used techniques, although several other chromatographic techniques can be applied [208-210]. These methods, coupled to mass spectrometry (MS), allow to a rapid and complete elucidation of the phenolic compounds structure.

2.5.2.1 Size exclusion chromatography

Size exclusion chromatography (SEC), in which the compounds are separated based on their molecular weight, can be a fast and simple tool to elucidate the size distribution of molecular weights of phenolic fractions. Some authors already applied this technique in

the characterisation of polar extracts of *E. globulus* [186, 211]. One disadvantage of SEC is that it does not allow the separation of individual components, but only groups of compounds with similar molecular weights. However, this can be interesting as a pre-fractionation technique for further analysis with other techniques, as matrix-assisted laser desorption ionisation–time of flight (MALDI-ToF), or even HPLC-MS, for example. In fact, Nonier *et al.*[209] reported a promise methodology in the analysis of proanthocyanidins, by using SEC to fractionate these compounds followed by MALDI-ToF analysis.

2.5.2.2 Gas chromatography

Gas chromatography (GC), in which the mobile phase is an inert gas, is a technique that can also be employed in the identification of phenolic compounds.

Despite some earlier studies have used this technique as the main tool in the identification of such compounds [212, 213], even in cork [55] its use is more recommended for the lower molecular weight phenolic compounds, due to their higher volatility [168]. In most cases, the detection of phenolic compounds is carried out using MS detector, although flame ionization detectors (FID) is also used to quantitative purposes [214]. One of the requirements and disadvantages of GC is the need of a previous derivatisation step. Phenolic compounds are converted into more volatile derivatives by methylation, trifluoroacetylation, trimethylsilylation or *tert*-butyldimethylsilylation [130]. GC-MS is a more powerful technique, given the spectroscopic information provided for each chromatographic peak, which in most cases is critical for its identification.

2.5.2.3 Thin layer chromatography

Thin layer chromatography (TLC) is a cheap and simple method to screen vegetal sources for the existence of different phenolic compounds. Furthermore, TLC can be used for preparative isolation, obtaining enriched fractions or pure compounds to further analysis by HPLC, GC or other spectroscopic techniques. Several advances were made, concerning the improvement of this technique, as, for example, the development of several strategies to identify specific class of compounds, as tannins or flavonoids [188, 215, 216]. Recently Sajewicz *et al.* [210] compared an improved TLC methodology with high-performance liquid chromatography (HPLC) in the identification of phenolic compounds from *Salvia* species, suggesting that, in some occasions, TLC could be a sufficient alternative to liquid chromatography. However, in general this technique is more commonly used in association with other techniques, such as HPLC. Conde *et al.* [38]

used them together in the identification of several phenolic compounds, including phenolic acids and flavonoids from *E. globulus* bark.

2.5.2.4 High-performance liquid chromatography

High-performance liquid chromatography (HPLC) is a chromatographic technique in which the stationary phase is composed of solid particles (such as silica, coated silica or polymers) packed into a column, and the mobile phase is a suitable liquid [217, 218].

This technique can be applied as normal phase (NP) or reverse-phase (RP) chromatography. NP involves a polar stationary phase and a non-polar mobile phase, being the more polar compounds retained longer into the column; Inversely, RP has a non-polar stationary phase and a polar mobile phase, with the less polar compounds being retained on the column longer than polar ones. This last type of chromatography is the most commonly applied. The interaction of the components with the mobile and stationary phase depends upon several parameters, thus can be easily manipulated. This allows HPLC to be a highly versatile technique and to have high ability to separate the components from a complex mixture. Furthermore, the analysis does not require a derivatisation step. In fact, in the last two decades HPLC has dominated the studies concerning the separation, quantification and identification of phenolic compounds. Several authors have demonstrated that it is possible to separate almost all the class of phenolic compounds (with the exception of higher tannins) in a single HPLC analysis [219-221].

The successful use of HPLC involves the choice of an appropriate column (stationary phase), mobile phases and detector. In the literature there are several reviews concerning the conditions of analysis used in different studies regarding phenolic compounds from vegetal sources [130, 168]; however, the variety of the phenolic fractions in each plant implies an individual screening of the optimal conditions for each one.

Columns

Reversed phases columns, in which silica particles are covered by non-polar alkane chains, are certainly the most used in the separation of phenolic compounds, with the C_{18} as the main choice for the stationary phase. Its internal diameter could range from 2.1 to 5 mm, however, when it is coupled with mass spectrometry, it is usual to move towards a lower diameter. Commonly, the length of the columns ranges from 100 to 300 mm and the particle size are mostly 3 or 5 µm [130, 168]. The choice of the columns to be used in the study of phenolic fraction from *Eucalyptus* spp. bark or *Q. suber* cork is also

unambiguous: a reversed-phase C_{18} column, with its length ranging from 150 to 200 mm and an internal diameter of 5 µm [38, 40, 43, 54, 55, 80].

The separation of tannins with high degree of polymerization is not yet possible by HPLC, due to the high number of isomers, resulting in large and unresolved chromatographic peaks [222]. However, it has been demonstrated that the use of a normal phase column is more suitable in the separation of these compounds. In fact, Lazarus *et al.* [223], separated proanthocyanidins, until dodecamers, from several food samples with a normal-phase column.

In the last years, several improvements have been done, both in instrumentation technology and in development of new columns, aiming to maximize the efficiency of separation, as also to decrease the analysis time. Ultra high-performance liquid chromatography (UHPLC) is an example of this, in which the equipments are suitable to support very high pressures (up to 1000 bar), allowing to use packed columns with a very low particle size (~2 µm) [224]. In fact, several authors have reported good separations of phenolic compounds in very short analysis times (less than 10 minutes) [225-227]. Recently, and to have alternatives to UHPLC, a still expensive and high solvent consumer technology, new HPLC columns were developed, allowing fast separation and high resolution with reduced mobile phase consumption. These, known as fused-core columns, due to its porous shell fused to a solid core, have demonstrated also promising results, allowing to separate phenolic compounds from teas and several other beverage extracts in less than 5 minutes [228]. However, due to their recent appearance in the market, very few studies have been done using these columns.

Mobile phases

The choice of appropriate solvent systems, as well the gradient elution, constitutes one of the main factors affecting the resolution and efficiency of HPLC. Furthermore, several factors have to be taken into account, such as the purity of the solvent, its viscosity or the compatibility with the detector. In reversed-phase chromatography the mobile phase is usually a mixture of water with an organic solvent. Methanol and acetonitrile are among the most used organic solvents for phenolic compounds separation [130, 168], being also the solvents chosen by several authors in the study the phenolic fraction from *Q. suber* cork [54, 80] and *Eucalyptus* bark. [38, 40, 43]. However, a good separation of phenolic compounds could also be achieved with other solvents, as propanol [229] or ethyl acetate [230]. The separation of the compounds can be done in isocratic mode, with the same mobile phase composition along the time or, as in the most cases, with a gradient elution

between two solvent mixtures. Frequently, various studies include a combination of the both, as it was applied by some authors to separate the phenolic compounds from *Q. suber* cork and from *E. globulus* bark [38, 43, 55, 80]. Nowadays, mostly of the HPLC analysis of phenolic compounds involve the addition of an acid to the mobile phase. Acetic, trifluoroacetic, phospohoric and formic acids are the mainly employed [130, 168]. Other studies reported the use of buffers in the preparation of the solvent mixtures [231].

Detectors

The choice of the appropriated detector is decisive in the success of an HPLC analysis. Several detectors are available in HPLC equipments, including ultraviolet-visible, photodiode array, fluorescence, electrochemical, conductivity and refractive index detectors, being the three first the most commonly used in the detection of phenolic compounds. Apart from the analysis done in these detectors, the identification of phenolic compounds is most frequently carried out by coupling mass spectrometry detectors to HPLC equipments.

Ultraviolet and Photodyode array detectors

Ultraviolet–visible (UV–Vis) spectrophotometry is one of the most common detectors in the analysis of phenolic compounds, due to the high UV absorption of these compounds. The appearance of diode array detectors (DAD) has become one of the most used techniques in these studies. DAD allows the detection and simultaneous recording of chromatograms at different wavelengths. Thus, different groups of phenolic compounds can be detected in complex matrixes. A match of both UV-vis spectrum and retention time with a reference compound could lead to a positive identification of a phenolic compound. In fact, several phenolic compounds were identified in *Q. suber* cork and *Eucalyptus* bark extracts by using HPLC-DAD and comparing the data with those of standard compounds [38, 54, 55, 80].

The choice of wavelengths for phenolic compounds detection also represents a crucial parameter affecting the success of HPLC-DAD, mainly for quantification purposes. Different classes of phenolic compounds can be detected by monitoring at different wavelengths, considering preferably those for which UV–Vis absorption is maximum (λmax). The λmax for benzoic acids, flavan-3-ols (including the dimers) and dihydrochalcones is near 280 nm, and for cinnamic acid and its derivatives is close to 320 nm. The λmax for flavonols is generally around 360 nm. Anthocyanins are one of the groups of phenolic compounds that can be easily detected, due to their absorption in

visible light, near 520 nm [232]. In the analysis of phenolic compounds from *Q. suber* cork, the wavelengths chosen range between 280 [80] and 325 nm [54, 55]. In the case of *Eucalyptus* bark extracts analysis, most of the authors have chosen 325 nm [38, 41]. Cadahía *et al.* [43] analysed the tannin composition of *Eucalyptus* spp. bark extracts by recording HPLC-DAD at 270 and 365 nm.

Fluorescence detector

Fluorescence detector (FL) is sensitive to compounds that fluoresce upon UV irradiation. It is considered more sensitive than other techniques, however, the application of this technique to the analysis of phenolic compounds can be limited if other strongly fluorescent components are present in the sample or even in the mobile phase.

Fluorescence detectors are most frequently used together with DAD in the analysis of phenolic compounds from vegetal sources, showing to be particularly sensitive to some compounds, as proanthocyanidins and catechin [223, 228]. This technique can also be applied in non-fluorescent compounds, provided an adequate derivatisation step is previously carried out [233]. Lores *et al.* [234] described a photochemical derivatisation step to detect some phenolic acids with a fluorescent detector.

Mass spectrometry detectors

Mass spectrometry (MS) can be used coupled directly to the HPLC, after other detectors, or off-line. In this last case, the fractions or pure compounds should be collected and further injected in spectrometer.

MS detectors manipulate and detect ions in the gaseous phase, therefore, the solvent has to be evaporated and sample ions must be produced [235]. This function is carried out by the interface of a MS detector, which can be divided in two main categories, the ion-spray techniques as electrospray ionization (ESI), thermospray (TSP) or atmospheric pressure chemical ionization (APCI) and the ion-desorption techniques as the matrix-assisted laser desorption ionisation (MALDI), fast atom bombardment (FAB) and plasma desorption (PD) [236]. ESI is the most commonly used ionization method for phenolic compounds [237], although some studies have been done using APCI interface. Most commercial HPLC-MS instruments can contain both interfaces, which can be operated both in positive or negative mode, however, negative mode is more frequent in the analysis of phenolic compounds [225, 237]. The sample solution enters into a MS detector flowing continuously trough a stainless steel capillary tube (ESI) or fused-silica capillary tube

(APCI). In an ESI interface a charge is added to the analytes, applying a potential in the capillary tip. After that, the sample is sprayed into the heated interface, where the solvent evaporates, assisted by a flow of hot nitrogen [235, 238]. In an APCI interface the solvent is vaporized first and, then, a charge is added to the analytes in the gaseous phase by using a corona discharge [235].

After sample ions are generated they are analysed according to their mass-to-charge ratio (*m*/*z*). There are also different mass separation devices, the so called mass analysers. The first analysers to be developed, and now the less used, were the magnetic-sector mass analysers, including the double-focusing and the tri-sector mass analysers [236]. Nowadays, the more common mass analysers are the quadrupole, the ion-trap and the time-of-flight (ToF), with the resolution, accuracy and mass range to be different between them [238].

Quadrupole, probably the most used type of mass analysers, consists of four parallel metal rods, in which direct-current and radio-frequency potentials are employed. Ion separation takes place varying the direct-current/radio-frequency ratio. For a given ratio, only selected *m*/*z* values can pass along the quadrupole to reach electron multiplier for detection. In an ion trap mass analyser, which consists of a ring shape electrode associated with end-cap electrodes, on the contrary, the ions are stored in the trapping space, and only specific *m/z* values are ejected for each direct-current/radio-frequency ratio [236, 238]. In ToF analysers a group of ions is accelerated, with a given kinetic energy, by an electric field. For the same kinetic energy the velocity of the ions will depend on their mass and, therefore, the time taken to transit the drift tube will be different. ToF combined with MALDI ionization has become widely used, especially in the analyses of higher molecular weight biomolecules [236, 238]. Vázquez *et al.* [40], for example, used this technique to identify gallotannins in *E. globulus* bark extracts.

One of the main advantages of mass spectrometry is that it supplies both information about molecular weight and structure of the analyte. The last one is achieved by the use of tandem mass spectrometry or mass spectrometry-mass spectrometry (MS/MS). This represents a technique in which, in a first stage, an ion from the analyte is isolated and, in a second stage, its fragmentation into product ions is promoted. There are several different MS/MS acquisition modes, being the product-ion scan the most usual. In this experiment the ion isolated in the first stage is submitted to a fragmentation [238]. Several instruments are available to perform tandem mass spectrometry being divided in tandem in-space and tandem in-line instruments.

In tandem in-space instruments the mass selection, fragmentation and mass analysis are carried out in three different regions of the mass spectrometer. Triple quadrupole (QqQ), hybrid mass spectrometer (instrument with two different types of mass analysers), tandem mass spectrometry on the ToF analyser and quadrupole-ToF instrument are examples of this type of equipments. QqQ is one of the most popular MS/MS equipments in the identification of phenolic compounds from vegetal sources [239, 240]. It consists of a set of three quadrupoles (Figure 2.26). The first and third quadrupoles act as mass analysers, while the second is used as collision cell. Therefore, an ion with a desired *m*/*z* can be isolated in the first quadrupole and sent to the second quadrupole (the collision cell), where it is fragmented. The product ions generated in this fragmentation pass to the third quadrupole, which can separate them (Figure 2.26).

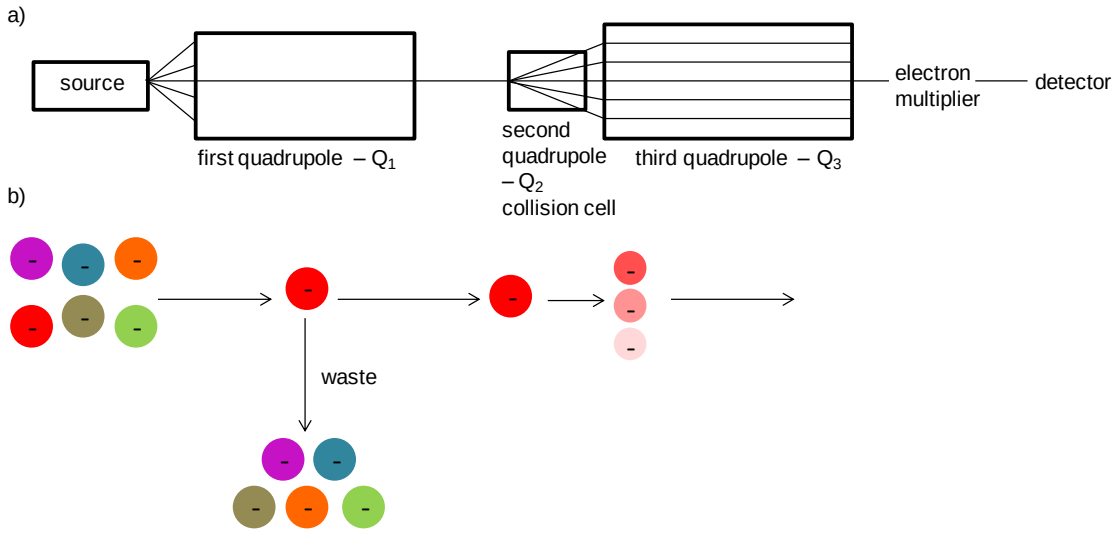


Figure 2.26 –Triple quadrupole mass spectrometer a) schematic (adapted from [241]); b) MS/MS experiment

In tandem in-time instruments the mass selection, fragmentation and analysis of ions is carried out in the same physical space but using a temporal sequence [238]. This type of tandem mass spectrometry is obtained in fourier transform ion cyclotron resonance (FT-ICR) or in ion trap mass spectrometers. The tandem mass spectrometry on an ion trap has a great advantage, since it is possible to isolate product ions and submit them to further fragmentation, known as multi-stage mass spectrometry (MS^n). After the ions are generated in the interface they go into the ion trap, where ions with an undesired *m*/*z* are sent to waste and the isolated ions further fragmented. Afterwards, product ions with a desired *m*/*z* can be held and fragmented into smaller molecules. This process can be performed over and over (Figure 2.27), depending of each ion-trap equipment, with a decreasing sensitivity between each fragmentation [235].

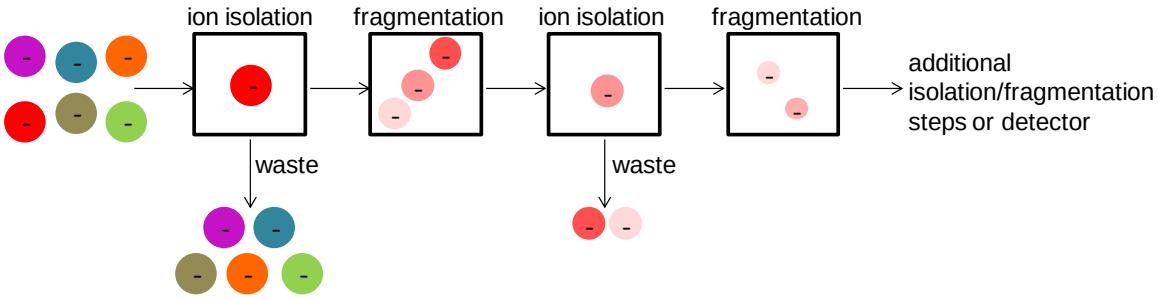


Figure 2.27 – MS^n experiment in an ion trap mass spectrometer

The ion trap mass spectrometers are of particular interest in the case of flavonoid glycosides, for example, since it is possible to isolate the aglycone and further fragment it, obtaining information about its structure, which is not possible with other instruments. Furthermore, this technique allows to elucidate the structure of other families of phenolic compounds. Fernandes *et al.* [80], for example, used an ion trap applying fragmentations until MS^3 to identify several gallic and ellagic acid derivatives, among others, in cork extracts.

2.5.2.5 Supercritical fluid chromatography

Supercritical fluid chromatography (SFC) is a recent technology similar to HPLC, which is compatible with most of the detectors used in HPLC, and even with those restricted to GC [242]. Several advantages have been attributed to SFC in comparison with HPLC, mostly due to the use of a supercritical fluid, as carbon dioxide. Properties, such as higher diffusivity and lower viscosity, result in lower separation time and higher resolution [232]. Despite all the advantages of SFC, this technique is not so explored in the separation of phenolic compounds, mostly due to the non-polar nature of carbon dioxide, the most used supercritical fluid. The separation of phenolic compounds can be achieved by the addition of polar modifiers, mostly methanol [243]. In order to reduce the amount of modifiers added to the mobile phase, some studies have been focused in the tuning of the polarity of the stationary phase, by using packed columns [244, 245]. The chromatographic separation can also be controlled by changing others parameters, such as the temperature and pressure [245].

2.6 Antioxidant activity analysis

Despite all the health benefits attributed to phenolic compounds, which were already described above, most of the studies involving the analysis of phenolic compounds from

natural sources include the evaluation of their antioxidant capacity. This is due, by on the one hand, to the antioxidant activity of natural compounds in human body, which is linked to the prevention of the most degenerative and oxidative stress related diseases [102]. On the other hand, most phenolic compounds have an antioxidant activity even higher than vitamin C and E [103]. Furthermore, the interest of phenolic compounds as food antioxidants is also related to concerns about the safety in the use of synthetic antioxidants, as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and *tert*-butylhydroquinone (TBHQ) [82, 246, 247].

In biological systems, an antioxidant can be defined as any substance that, in low concentration, compared to those of an oxidisable substrate, significantly delays or prevents oxidation of that substrate [248]. The substrate, i.e. the oxidisable compound, is usually a lipid, but can also be a protein, deoxyribonucleic acid (DNA) or carbohydrate. In the case of lipid oxidation, the main mechanism of antioxidants is to act as radical chain-breakers. Another mechanism is to act as preventive antioxidant oxygen scavenging or blocking the pro-oxidant effects, by binding proteins that contain catalytic metal sites [249].

The antioxidant activity of phenolic compounds is mainly due to their reducing properties, allowing them to act as hydrogen or electron donators. Furthermore, they have transition metal-chelation potential [103]. There is a structure-activity relationship between the phenolic compounds that relates the radical scavenging with the presence, number or position of substitution groups [82]. It was reported that the number of hydroxyl groups in phenolic acids increases their antioxidant capacity, as well as the methoxy substitution in *ortho* position to the hydroxyl group of monohydroxycinnamic acids increases the antioxidant activity of such compounds [250]. In the same way, it was verified that the *O*-substitution on B ring of flavonoids also increase their antioxidant activity against lipid radicals [250]. A double bond between C-2 and C-3, conjugated with a 4-oxo group on C ring also increases the antioxidant capacity of flavonoids [251]. In the case of gallotannins, the increase of the number of galloyl units increases the antioxidant activity. It was also verified that the antioxidant capacity of ellagitannins increases with the molecular weight of the compounds [252]. Actually, Fernandes *et al.* [148] verified the same behaviour with gallo- and ellagitannins isolated from *Q*. *suber* cork.

Numerous *in vitro* methods have been proposed to measure the antioxidant activity of pure compounds or vegetal extracts, which can be divided in two main groups: those based on hydrogen atom transfer (HAT) reactions and those based on single electron

transfer (ET) reactions. HAT assays measure the capacity of a potential antioxidant to donate hydrogen atoms and quench free radicals:

$$X^{\cdot} + AH \rightarrow XH + A^{\cdot} \quad (2.1)$$

Generally, HAT reactions involve a synthetic free radical generator, an oxidisable molecular probe and antioxidant compounds. Most of the HAT assays are kinetic-based, involving a competition between the antioxidant and the substrate for peroxyl radicals, resulted from the decomposition of azo compounds [253]. The HAT reactions are quite fast and independent of pH and solvent [180].

The ET-based assays measure the capacity of a potential antioxidant to transfer any electron to an oxidant, which itself is the probe, changing its colour when reduced [253]. The oxidant can be metals, radicals or carbonyls:

$$X^{\cdot} + AH \rightarrow X^{-} + AH^{\cdot +} \quad (2.2)$$

$$AH^{\cdot +} \overset{H_2O}{\longleftrightarrow} A^{\cdot} + H_3O^{+} \quad (2.3)$$

$$X^{-} + H_3O^{+} \rightarrow XH + H_2O \quad (2.4)$$

$$M(III) + AH \rightarrow AH^{+} + M(II) \quad (2.5)$$

Contrary to HAT, ET reactions are slow and pH dependent [180].

The assays based on each mechanism most used in the analysis of phenolic compounds will be briefly described:

2.6.1 Hydrogen atom transfer-based assays

2.6.1.1 Oxygen radical absorbance capacity

The oxygen radical absorbance capacity (ORAC) has been used to evaluate the antioxidant capacity in human and animal systems, however, in the last years, its use has been extended to other matrixes, as in the food industry. In fact, some nutraceutical manufacturers began to include ORAC values on their product labels [180].

This assay, developed by Cao *et al.* [254], measures the capacity of antioxidant compounds to compete with a fluorescent probe, such as fluorescein, for a thermally generated peroxyl radical. While the reaction of fluorescein with the peroxyl radical occurs, fluorescein is consumed and, consequently, the fluorescence intensity decreases:

$$R\text{-}N{=}N\text{-}R \xrightarrow{O_2} N_2 + 2ROO^{\cdot} \quad (2.6)$$

$$ROO^{\cdot} + probe \rightarrow ROOH + oxidized\ probe \quad (2.7)$$

$$ROO^{\cdot} + AH \rightarrow ROOH + A^{\cdot} \quad (2.8)$$

$$ROO^{\cdot} + A^{\cdot} \rightarrow ROOA \quad (2.9)$$

The antioxidant capacity of the sample is quantified by the area under the fluorescence decay curve (relative fluorescence intensity of the probe vs time). Trolox, a commercial water-soluble vitamin E derivative (Figure 2.28), is commonly used as a standard compound, to obtain calibration curves and being the ORAC values expressed as Trolox equivalents.

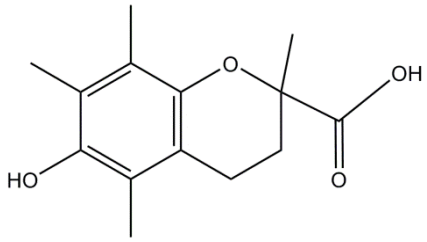


Figure 2.28 – Structure of trolox

The major limitation attributed to this assay is related to the long period of analysis [180]. Several studies concerning the antioxidant activity of extracts rich in phenolic compounds have been done using this assay [104, 145].

2.6.1.2 Total peroxyl radical-trapping antioxidant parameter

Total peroxyl radical-trapping antioxidant parameter (TRAP) assay is based on the monitoring of the reaction between the peroxyl radicals, generated from 2,2‘-azobis(2-amidinopropane) dihydrochloride (AAPH), and the target probes. This assay could use fluorescence, with R-phycoerythrin (R-PE), or absorbance, by the use of 2,2‘-azinobis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), as reaction probes [180, 253]. The antioxidant capacity is commonly quantified by the lag time or reaction time, being trolox generally the reference compound used. One of the main criticisms to this assay is related to its complexity, time-consuming and the possible underestimation, due to be based on the lag phase of antioxidant compounds [180]. This assay is commonly used in the analysis of phenolic compounds fractions, together with other assays [255].

2.6.1.3 *β*-carotene bleaching assay

β-carotene bleaching assay is based on the reaction of *β*-carotene with peroxyl radicals (e.g. AAPH or oxidizing lipids), generating *β*-carotene epoxydes [256]. The antioxidant compounds compete with the radical, donating it hydrogen atoms [180], being their content monitored by controlling the *β*-carotene colour, which has an absorption maximum at 470 nm. Other form of this method, known by croton assay, uses crucin instead *β*-carotene, however, this natural compound is not commercially available [180]. This assay has been used by several authors to analyse the antioxidant activity of extracts rich in phenolic compounds [257, 258].

2.6.2 Single electron transfer-based assays

2.6.2.1 Ferric reducing antioxidant power assay

Ferric reducing antioxidant power (FRAP) assay, firstly developed by Benzie [259] to quantify ascorbic acid in plasma, is based on the measurement, under acidic conditions, of the reduction of Fe^{3+} – 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) complex to Fe^{2+} form (Figure 2.29), which has an intense blue colour.

Despite being considered a fast and simple method to evaluate the antioxidant activity of phenolic compounds, one of the main disadvantages of this assay is related to the different reactivity between those compounds along the time [180]. Nevertheless, this method has been applied to investigate the antioxidant activity of phenolic fractions from many natural products, such as in fruits [196], vegetables [260] or even in *E. globulus* bark [29]. Furthermore, this assay was also used to evaluate the antioxidant activity of phenolic compounds isolated from *Q. suber* cork [148].

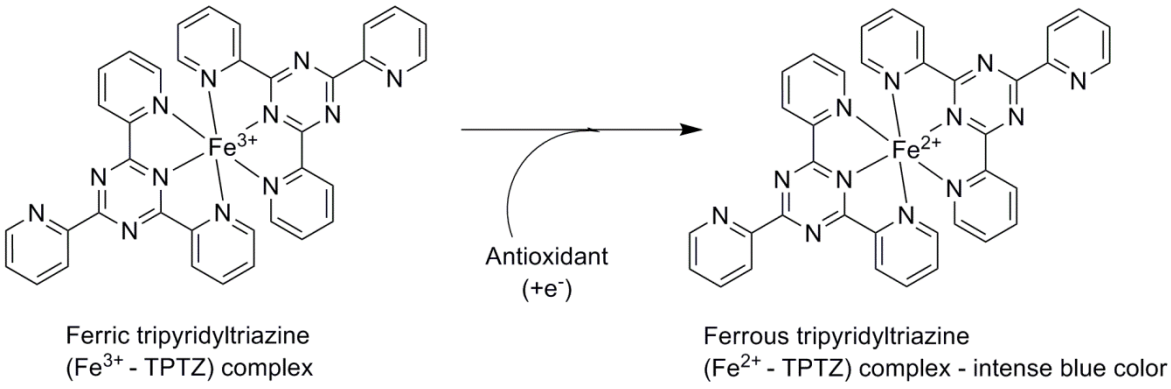


Figure 2.29 – Reaction mechanism between Fe^{3+}-TPTZ complex and antioxidant compounds (source [256])

2.6.2.2 Copper reducing assay

Copper reducing assay is a recent variant of the FRAP assay, which is based on the reduction of Cu (II) to Cu (I) by the action of the reducing agents (antioxidants) presented in the sample. There are two forms of this method: the AOP-490 [261], in which a chromogenic reagent is used, namely bathocuproine (2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline), to form a complex with CU (I) with a maximum of absorbance at 490 nm; and the CUPRAC assay [262], which uses neocuproine (2,9-dimethyl-1,10-phenanthroline). This compound complex with CU (I), forming a chromophore with a maximum absorbance at 450 nm. Uric acid is commonly used as reference compound. One of the main disadvantages attributed to this assay is, as occur with the FRAP method, related to the selection of the proper reaction time [180]. However, several determinations of the antioxidant activity of phenolic fractions have been done using this assay [258, 260].

2.6.2.3 Trolox equivalents antioxidant capacity assay

Trolox equivalents antioxidant capacity (TEAC) assay, also known by 2,2′-Azinobis (3-ethylbenzothiazoline-6 sulfonic acid) (ABTS) assay, was firstly reported by Miller and co-workers [263]. In the improved version of this assay [264] a stable ABTS radical cation is generated by oxidation of ABTS with potassium persulfate. Therefore, the antioxidant compounds will react directly with $ABTS^{\bullet+}$, which has a blue-green chromophore absorption (Figure 2.30). The antioxidant capacity is measured by monitoring the decreasing of the intense colour of the radical cation. Trolox is commonly used as the referenced compound, and the final results reported as trolox equivalents. This assay has been used by several authors to investigate the natural antioxidants, alone [109, 179], or in combination with other methods, as FRAP [265] or DPPH assays [266].

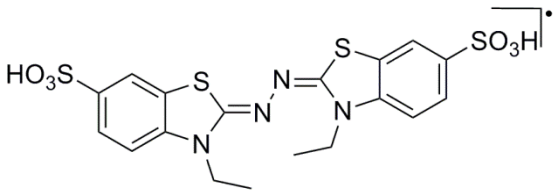


Figure 2.30 – Structure of 2,2′-Azinobis (3-ethylbenzothiazoline-6 sulfonic acid) radical cation ($ABTS^{\bullet+}$)

2.6.2.4 2,2-diphenyl-1-picrylhydrazyl assay

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, originally described by Brand-Williams *et al.* [267], is used worldwide to analyse the free radical scavenging activity. Actually, a brief search on ISI-Web of Knowledge (Figure 2.31) shows the tremendous difference between the number of studies using this assay compared with those described before.

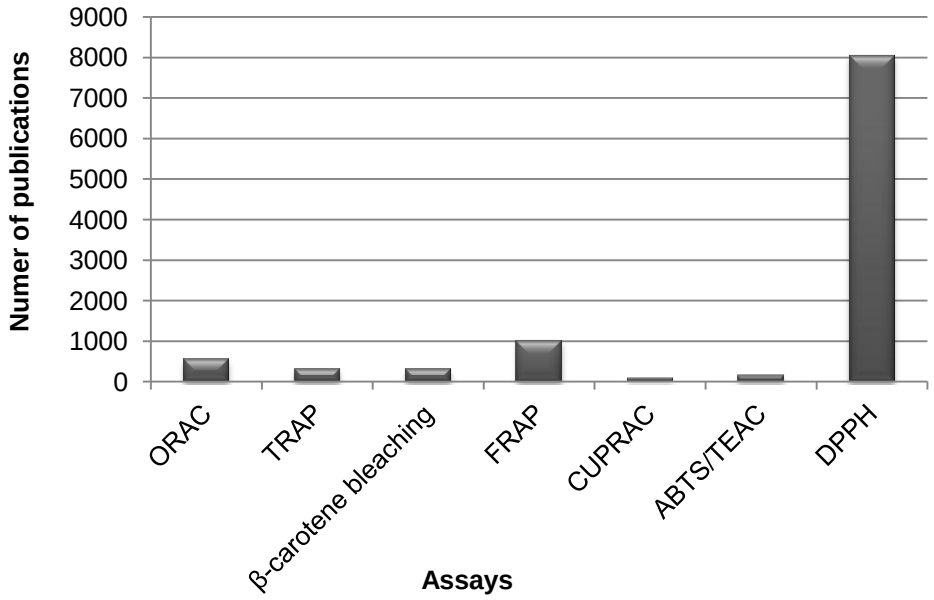


Figure 2.31 – Number of publication, until today, considering the keywords in the topics search "phenolic", "antioxidant" and respectively: "ORAC"; "TRAP"; "carotene bleaching"; "FRAP"; CUPRAC"; "ABTS TEAC"; and DPPH" (ISI-Web of Knowledge-October 2012)

The method is based on the colour decrease of the DPPH free radical solution, and it is mostly described as an ET-based assay, although some authors defended that DPPH, as TEAC assays, use both HAT and ET mechanisms [180, 268]. Moon and Shibamoto [256] described that the odd electron of the nitrogen atom in DPPH$^{\bullet}$ is reduced by receiving a hydrogen atom from antioxidant compounds (Figure 2.32). However, Foti and co-workers [269] reported that the rate-determining mechanism in this assay, in a methanol or ethanol solution, is a fast electron transfer process, while the HAT becomes a marginal reaction path.

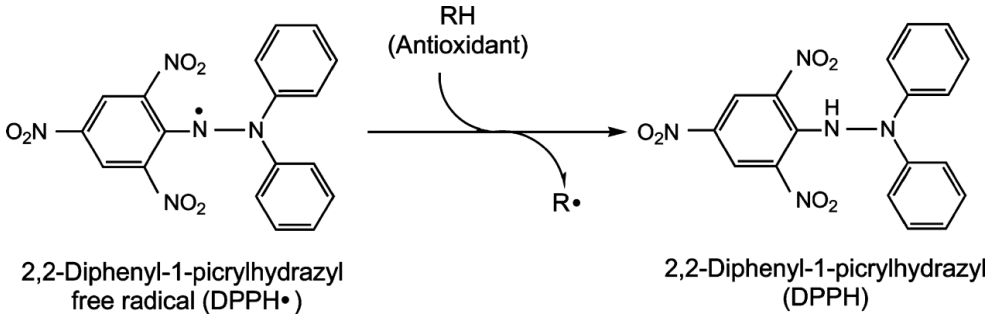


Figure 2.32 – Proposed reaction mechanism between DPPH$^{\bullet}$ and antioxidant compounds (source [256])

The radical has maximum UV absorption at 515/517 nm and the colour fade can be monitored easily by a spectrophotometer. The final results are expressed as IC_{50} values, which correspond to the compound or extract concentrations need to reduce in 50 % the initial DPPH radical concentration. Ascorbic acid and BHT (Figure 2.33), two of the main commercial antioxidants, are the mainly reference compounds used in this assay.

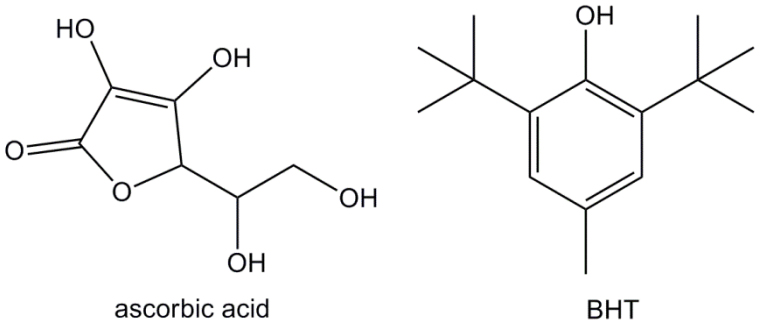


Figure 2.33 – Structures of ascorbic acid and BHT

DPPH$^{\bullet}$ is recognized as a stable free radical, however, it is light, oxygen and pH sensitive [270]. The type of the solvent used also affect the success of the method, mainly due to the solubility of the radical. Another parameter affecting the final results is the DPPH$^{\bullet}$ concentration [271]. Nevertheless, there are several studies using a wide diversity of conditions. Sharma and Bhat [272] recommended a DPPH$^{\bullet}$ concentration of 50µM in methanol or buffered methanol [257], as well as other parameters, such as the incubation time or the absence of light. Furthermore, to overcome the non existence of a standardised procedure, Scherer and Godoy [271] suggested the use of a ratio between the DPPH concentration and the IC_{50} value, called antioxidant activity index, however, very few studies have used this ratio [273-275]. One of the main advantages of this assay is that the DPPH$^{\bullet}$ does not require any previous preparation, as happens with the ABTS radical, for example.

This assay was already used, together with FRAP, in the analysis of the antioxidant activity of phenolic compounds present in cork [148]. El-Moein and co-workers [276] also used DPPH, but in association with *β*-carotene and ABTS assays, to evaluate the antioxidant activity of extracts from *E. globulus* bark. Although some other studies have used DPPH combined with other assays [152, 260], a large number of authors have used only DPPH assay to access the antioxidant properties of the phenolic fraction from vegetal extracts [162, 225, 244, 273, 274].

References

[1] Pinho, J., 5º Inventário Nacional Florestal. In Direção Nacional de Gestão Florestal, Ed. 2012; Vol. 2012.

[2] Ugalde, L.; Pérez, O. Mean annual volume increment of selected industrial forest plantation species. www.fao.org/forestry (September 28, 2012)

[3] Trabado, G. I.; Wilstermann, D. Cultivated *Eucalyptus* Forests Global Map 2008 Version 1.0.1. http://www.git-forestry.com (September 11, 2012)

[4] Ladiges, P.; Wasshausen, D., Eucalypt domestication and breeding. *Brittonia* **1996,** *48*, 494-494.

[5] Potts, B. M.; Vaillancourt, R. E.; Jordan, G.; Dutkowski, G.; Silva, J.; McKinnon, G.; Steane, D.; Volker, P.; Lopez, G.; Apiolaza, L.; Li, L. N.; Marques, C.; Borralho, N. In *Eucalyptus in a changing world*, IUFRO Conference, Aveiro, Portugal, 11-15 October, 2004; Borralho, N.; Pereira, J. S.; Marques, C.; Coutinho, J.; Madeira, M.; Tomé, M., Eds. Aveiro, Portugal, 2004.

[6] Myburg, Z.; Grattapaglia, D.; Potts, B.; Labate, C.; Bossinger, B.; Byrne, M.; Vaillancourt, R.; Sederoff, R.; Southerton, S., Sequencing of the *Eucalyptus* genome: A proposal to DOE-JGI. In The US Department of Energy Joint Genome Institute, Ed. 2006.

[7] Quilhó, T.; Miranda, I.; Pereira, H., Within-tree variation in wood fibre biometry and basic density of the urograndis eucalypt hybrid (*Eucalyptus grandis* x *E. urophylla*). *IAWA J.* **2006,** *27*, 243-254.

[8] ABRAF. ABRAF Statistical yearbook - base year 2010. http://www.abraflor.org.br (September 19, 2012)

[9] Kibblewhite, R. P.; Johnson, B. I.; Shelbourne, C. J. A. In *Kraft pulp qualities of Eucalyptus nitens, E. globulus, and E. maidenii, at ages 8 and 11 years*, 55th Appita Annual Conference Proceedings, Hobart, Australia, 2001; Hobart, Australia, 2001.

[10] Lopez, G.; Potts, B.; Rodriguez, J. T.; Gelid, P. In *The performance of Eucalyptus maidenii provenances in Argentina*, IUFRO International symposium-Developing the Eucalyptus of the Future Valdivia, Chile, 10-15 September, 2001; Valdivia, Chile, 2001.

[11] Li, D. Q.; Liu, Y. P.; Zheng, H. S.; Zhu, R. G.; Huang, Y. X., Analysis of genetic effects on growth traits in Eucalyptus *maidenii* F. Muell. In *Eucalyptus Plantations: Research , Management and Development*, Wei, R. P.; Xu, D., Eds. World Scientific: Singapore, 2003; pp 229-238.

[12] Quilhó, T.; Pereira, H., Within and between-tree variation of bark content and wood density of *Eucalyptus globulus* in commercial plantations. *IAWA J.* **2001,** *22*, 255-265.

[13] APCOR (Associação Portuguesa da Cortiça). 2011 Yearbook. http://www.realcork.org/userfiles/File/Publicacoes/anuario2011.pdf (September 19, 2012)

[14] Gil, L., Cork as a Building Material.Technical Manual. In APCOR – Portuguese Cork Association, Ed. http://www.apcor.pt/userfiles/File/Caderno%20Tecnico%20F%20EN.pdf, 2007.

[15] Silva, S. P.; Sabino, M. A.; Fernandes, E. M.; Correlo, V. M.; Boesel, L. F.; Reis, R. L., Cork: properties, capabilities and applications. *Int. Mater. Rev.* **2005,** *50*, 345-365.

[16] Fortes, M. A.; Rosa, M. E.; Pereira, H., *A cortiça*. IST Press: Lisbon, 2004.

[17] Pereira, H., *Cork: Biology, Production and Uses: Biology, Production and Uses*. Elsevier Amsterdam, 2007.

[18] Gil, L., *Cortiça: produção, tecnologia, e aplicação*. INETI: Lisbon, 1998.

[19] Pintor, A. M. A.; Ferreira, C. I. A.; Pereira, J. C.; Correia, P.; Silva, S. P.; Vilar, V. J. P.; Botelho, C. M. S.; Boaventura, R. A. R., Use of cork powder and granules for the adsorption of pollutants: A review. *Water Res.* **2012,** *46*, 3152-3166.

[20] Fengel, D.; Wegener, G., *Wood: chemistry, ultrastructure, reactions*. Walter de Gruyter, Co.: Berlin, 1984.

[21] Kurth, E. F., The chemical composition of barks. *Chem. Rev.* **1947,** *40*, 33-49.

[22] Ek, M.; Gellerstedt, G.; Henriksson, G., Wood chemistry and wood biotechnology. In *Pulp and Paper Chemistry and Technology*, Walter de Gruyter: Berlin, 2009; Vol. 1.

[23] Sakai, K., Chemistry of bark. In *Wood and cellulosic chemistry*, 2nd ed.; Hon, D. N.-S.; Shiraishi, N., Eds. Marcel Dekker, Inc.: Basel, Switzerland, 2001; pp 243-274.

[24] Bennett, R. N.; Wallsgrove, R. M., Secondary metabolites in plant defense-mechanisms. *New Phytol.* **1994,** *127*, 617-633.

[25] Mirra, I. M. P. Influência das diferentes granulometrias na composição química das cascas de *Eucalyptus globulus* Labill., *Betula pendula* Roth, *Picea abies* (L.) Karst., *Pinus sylvestris* L. e *Pinus pinea* L. Universidade Técnica de Lisboa, Lisbon, 2011.

[26] Miranda, I.; Gominho, J.; Mirra, I.; Pereira, H., Fractioning and chemical characterization of barks of *Betula pendula* and *Eucalyptus globulus*. *Ind. Crop. Prod.* **2013,** *41*, 299-305.

[27] Miranda, I.; Gominho, J.; Pereira, H., Incorporation of bark and tops in *Eucalyptus globulus* wood pulping. *BioResources* **2012,** *7*, 4350-4361.

[28] Pereira, H., Variability in the chemical composition of plantation eucalypts (*Eucalyptus globulus* Labill). *Wood Fiber Sci.* **1988,** *20*, 82-90.

[29] Vázquez, G.; Fontenla, E.; Santos, J.; Freire, M. S.; González-Álvarez, J.; Antorrena, G., Antioxidant activity and phenolic content of chestnut (*Castanea sativa*) shell and eucalyptus (*Eucalyptus globulus*) bark extracts. *Ind. Crop. Prod.* **2008,** *28*, 279-285.

[30] Bargatto, J. Avaliação do potencial da casca de *Eucalyptus* spp. para a produção de bioethanol. Universidade de S. Paulo, Piracicaba, Brazil, 2010.

[31] Freire, C. S. R.; Silvestre, A. J. D.; Neto, C. P.; Cavaleiro, J. A. S., Lipophilic extractives of the inner and outer barks of *Eucalyptus globulus*. *Holzforschung* **2002,** *56*, 372-379.

[32] Domingues, R. M. A.; Sousa, G. D. A.; Silva, C. M.; Freire, C. S. R.; Silvestre, A. J. D.; Neto, C. P., High value triterpenic compounds from the outer barks of several Eucalyptus species cultivated in Brazil and in Portugal. *Ind. Crop. Prod.* **2011,** *33*, 158-164.

[33] Kolattukudy, P. E., Biochemistry and function of cutin and suberin. *Can. J. Bot.-Rev. Can. Bot.* **1984,** *62*, 2918-2933.

[34] Bernards, M. A., Demystifying suberin. *Can. J. Bot.-Rev. Can. Bot.* **2002,** *80*, 227-240.

[35] Domingues, R. M. A.; Sousa, G. D. A.; Freire, C. S. R.; Silvestre, A. J. D.; Pascoal Neto, C., *Eucalyptus globulus* biomass residues from pulping industry as a source of high value triterpenic compounds. *Ind. Crop. Prod.* **2010,** *31*, 65-70.

[36] Yun, B.-S.; Lee, I.-K.; Kim, J.-P.; Chung, S.-H.; Shim, G.-S.; Yoo, I.-D., Lipid peroxidation inhibitory activity of some constituents isolated from the stem bark of *Eucalyptus globulus*. *Arch. Pharm. Res.* **2000,** *23*, 147-150.

[37] Banthorpe, D. V., Terpenoids. In *Natural Products: Their Chemistry and Biological Significance*, Mann, J.; Davidson, R. S.; Hobbs, J. B.; Banthorpe, D. V.; Harborne, J. B., Eds. Longman Scientific & Technical: United Kingdom, 1994; pp 289-358.

[38] Conde, E.; Cadahía, E.; García-Vallejo, M.; Tomás-Barberán, F., Low molecular weight polyphenols in wood and bark of *Eucalyptus globulus Wood Fiber Sci.* **1995,** *27*, 379-383.

[39] Yazaki, Y.; Hillis, W. E., Polyphenols of *Eucalyptus globulus*, *E. Regnans* and *E. Deglupta*. *Phytochemistry* **1976,** *15*, 1180-1182.

[40] Vázquez, G.; Santos, J.; Freire, M.; Antorrena, G.; González-Álvarez, J., Extraction of antioxidants from eucalyptus (*Eucalyptus globulus*) bark. *Wood Sci. Technol.* **2012,** *46*, 443-457.

[41] Conde, E.; Cadahia, E.; DiezBarra, R.; GarciaVallejo, M. C., Polyphenolic composition of bark extracts from *Eucalyptus camaldulensis*, *E. globulus* and *E. rudis*. *Holz Als Roh-und Werkst.* **1996,** *54*, 175-181.

[42] Kim, J. P.; Lee, I. K.; Yun, B. S.; Chung, S. H.; Shim, G. S.; Koshino, H.; Yoo, I. D., Ellagic acid rhamnosides from the stem bark of *Eucalyptus globulus*. *Phytochemistry* **2001,** *57*, 587-591.

[43] Cadahía, E.; Conde, E.; deSimon, B. F.; GarciaVallejo, M. C., Tannin composition of *Eucalyptus camaldulensis*, *E. globulus* and *E. rudis*. Part II. Bark. *Holzforschung* **1997,** *51*, 125-129.

[44] Fechtal, M.; Riedl, B., Analyse des extraits tannants des écorces des *Eucalyptus* après hydrolyse acide par la chromatographie en phase gazeuse couplée avec la spectrométrie de masse (GC-MS). *Holzforschung* **1991,** *45*, 269-273.

[45] Pereira, H., Chemical composition and variability of cork from *Quercus suber* L. *Wood Sci.Technol.* **1988,** *22*, 211-218.

[46] Pereira, H., Composição química da cortiça virgem e da cortiça de reprodução amadia do *Quercus suber* L. *Bol. Inst. Prod. Flor. - Cortiça* **1984,** *550*, 237-240.

[47] Pinto, P. C. R. O.; Sousa, A. R.; Silvestre, A. J. D.; Neto, C. P.; Gandini, A.; Eckerman, C.; Holmbom, B., Quercus suber and Betula pendula outer barks as renewable sources of oleochemicals: A comparative study. *Ind. Crop. Prod.* **2009,** *29*, 126-132.

[48] Gandini, A.; Pascoal Neto, C.; Silvestre, A. J. D., Suberin: A promising renewable resource for novel macromolecular materials. *Prog. Polym. Sci.* **2006,** *31*, 878-892.

[49] Silvestre, A. J. D.; Neto, C. P.; Gandini, A., Cork and Suberins: Major Sources, Properties and Applications. In *Monomers, Polymers and Composites from Renewable Resources*, Mohamed Naceur, B.; Alessandro, G., Eds. Elsevier: Amsterdam, 2008; pp 305-320.

[50] Sousa, A. F.; Gandini, A.; Silvestre, A. J. D.; Pascoal Neto, C.; Cruz Pinto, J. J. C.; Eckerman, C.; Holmbom, B., Novel Suberin-Based Biopolyesters: From Synthesis to Properties. *J. Polym. Sci. Pol. Chem.* **2011,** *49*, 2281-2291.

[51] Castola, V.; Marongiu, B.; Bighelli, A.; Floris, C.; Lai, A.; Casanova, J., Extractives of cork (*Quercus suber* L.): chemical composition of dichloromethane and supercritical CO_2 extracts. *Ind. Crop. Prod.* **2005,** *21*, 65-69.

[52] Sousa, A. F.; Pinto, P. C. R. O.; Silvestre, A. J. D.; Neto, C. P., Triterpenic and other lipophilic components from industrial cork byproducts. *J. Agric. Food Chem.* **2006,** *54*, 6888-6893.

[53] Conde, E.; Garcia-Vallejo, M. C.; Cadahia, E., Waxes composition of *Quercus suber* reproduction cork from different Spanish provenances. *Wood Sci.Technol.* **1999,** *33*, 271-283.

[54] Conde, E.; Cadahia, E.; Garcia-Vallejo, M. C.; de Simon, B. F., Polyphenolic composition of *Quercus suber* cork from different Spanish provenances. *J. Agric. Food Chem.* **1998,** *46*, 3166-3171.

[55] Conde, E.; Cadahía, E.; García-Vallejo, M. C.; de Simón, B. F.; Adrados, J. R. G., Low molecular weight polyphenols in cork of *Quercus suber*. *J. Agric. Food Chem.* **1997,** *45*, 2695-2700.

[56] Pena-Neira, A.; Hernandez, T.; Garcia-Vallejo, M. C.; Cadahia, E.; de Simon, B. F.; Suarez, J. A., Low molecular weight phenols in cork stoppers. *Am. J. Enol. Viticult.* **1999,** *50*, 285-290.

[57] Pena-Neira, A.; Bartolome, B.; Cadahia, E.; Hernandez, T.; Garcia-Vallejo, C.; Gonzalez-Adrados, J. R.; Suarez-Lepe, J. A., Low molecular-weight phenolic compounds in natural and agglomerated cork stoppers. *Sci. Aliment.* **1999,** *19*, 119-124.

[58] Mazzoleni, V.; Caldentey, P.; Silva, A., Phenolic compounds in cork used for production of wine stoppers as affected by storage and boiling of cork slabs. *Am. J. Enol. Viticult.* **1998,** *49*, 6-10.

[59] Pereira, H., Chemical composition of cork from *Quercus suber* L. *Anais Inst. Sup. Agron.* **1982,** *Vol. XL*, 17-25.

[60] Caldas, M. M.; Ferreira, J. L.; Borges, M., Boletim do instituto dos produtos florestais. *Cortiça* **1986,** *578*, 339-342.

[61] Carvalho, J. S., A química da cortiça. *Bol. Junta Nac. Cortiça* 1968, pp 3-11.

[62] Pereira, H., Constituição química da cortiça - Estado actual dos conhecimentos. *Cortiça* **1979,** *483*, 259-264.

[63] Holloway, P. J., The suberin composition of the cork layers from some ribes species. *Chem. Phys. Lipids* **1972,** *9*, 171-179.

[64] Parameswaran, N.; Liese, W.; Günzerodt, H., Características do verde da cortiça de *Quercus suber*. *Cortiça* **1981,** *514*, 179-184.

[65] Conde, E.; Cadahía, E.; García-Vallejo, M. C.; González-Adrados, J. R., Chemical characterization of reproduction cork from spanish *Quercus suber*. *J. Wood Chem. Technol.* **1998,** *18*, 447-469.

[66] Jové, P.; Olivella, M. A.; Cano, L., Cork bark layer composition. *BioResources* **2011,** *6*, 1806-1815.

[67] Asensio, A., Structural studies of the hemicellulose A from the cork of *Quercus suber*. *Carbohyd. Res.* **1987,** *161*, 167-170.

[68] Asensio, A., Structural studies of a hemicellulose B fraction from the cork of *Quercus suber*. *Carbohyd. Res.* **1987,** *165*, 134-138.

[69] Asensio, A., Structural studies of a hemicellulose B fraction (B-2) from the cork of Quercus suber. *Can. J. Chemistry* **1988,** *66*, 449-453.

[70] Marques, A. V.; Pereira, H.; Meier, D.; Faix, O., Structural characterization of cork lignin by thioacidolysis and permanganate oxidation. *Holzforschung* **1999,** *53*, 167-174.

[71] Akim, L. G.; Cordeiro, N.; Neto, C. P.; Gandini, A., Cork lignin characterization with dry hydrogen iodide H-1 NMR/GPC analysis: A comparative study. *Abstr. Pap. Am. Chem. Soc.* **1998,** *215*, U136-U136.

[72] Cordeiro, N.; Belgacem, M. N.; Silvestre, A. J. D.; Neto, C. P.; Gandini, A., Cork suberin as a new source of chemicals. 1. Isolation and chemical characterization of its composition. *Int. J. Biol. Macromol.* **1998,** *22*, 71-80.

[73] Lopes, M. H.; Neto, C. P.; Barros, A. S.; Rutledge, D.; Delgadillo, I.; Gil, A. M., Quantitation of aliphatic suberin in *Quercus suber* L. cork by FTIR spectroscopy and solid-state C-13-NMR spectroscopy. *Biopolymers* **2000,** *57*, 344-351.

[74] Conde, E.; García-Vallejo, M. C.; Cadahía, E., Variability of suberin composition of reproduction cork from *Quercus suber* throughout industrial processing. *Holzforschung* **1999,** *53*, 56-62.

[75] Lopes, M. H.; Gil, A. M.; Silvestre, A. J. D.; Neto, C. P., Composition of Suberin Extracted upon Gradual Alkaline Methanolysis of Quercus suber L. Cork. *J. Agric. Food Chem.* **2000,** *48*, 383-391.

[76] Cordeiro, N.; Belgacem, M. N.; Silvestre, A. J. D.; Pascoal Neto, C.; Gandini, A., Cork suberin as a new source of chemicals.: 1. Isolation and chemical characterization of its composition. *Int. J. Biol. Macromol.* **1998,** *22*, 71-80.

[77] Bescansa-López, J. L.; Ribas-Marqués, I., Quimica del corcho.16: Hidrocarburos parafinicos en el corcho. *An. Soc. Esp. Fis.-Quim.* **1966,** *62-B*, 871-873.

[78] Conde, E.; Garcia-Vallejo, M. C.; Cadahia, E., Waxes composition of reproduction cork from *Quercus suber* and its variability throughout the industrial processing. *Wood Sci. Technol.* **1999,** *33*, 229-244.

[79] Cadahia, E.; Conde, E.; de Simon, B. F.; Garcia-Vallejo, M. C., Changes in tannic composition of reproduction cork *Quercus suber* throughout industrial processing. *J. Agric. Food Chem.* **1998,** *46*, 2332-2336.

[80] Fernandes, A.; Sousa, A.; Mateus, N.; Cabral, M.; de Freitas, V., Analysis of phenolic compounds in cork from *Quercus suber* L. by HPLC–DAD/ESI–MS. *Food Chem.* **2011,** *125*, 1398-1405.

[81] Varea, S.; Garcia-Vallejo, M. C.; Cadahia, E.; de Simon, B. F., Polyphenols susceptible to migrate from cork stoppers to wine. *Eur. Food Res. Technol.* **2001,** *213*, 56-61.

[82] Balasundram, N.; Sundram, K.; Samman, S., Phenolic compounds in plants and agri-industrial by-products: Antioxidant activity, occurrence, and potential uses. *Food Chem.* **2006,** *99*, 191-203.

[83] Kefeli, V. I.; Kalevitch, M. V.; borsari, B., Phenolic cycle in plants and environment. *J. Cell Mol. Biol.* **2003,** *2*, 13-18.

[84] Bravo, L., Polyphenols: Chemistry, dietary sources, metabolism, and nutritional significance. *Nutr. Rev.* **1998,** *56*, 317-333.

[85] Vermerris, W.; Nicholson, R., Families of Phenolic Compounds and Means of Classification. In *Phenolic Compound Biochemistry*, Springer Netherlands: 2006; pp 1-34.

[86] Harborne, J. B.; Simmonds, N. W., *Biochemistry of phenolic compounds*. Academic Press: London, 1964.

[87] Shahidi, F.; Naczk, M., *Food Phenolics: Sources, Chemistry, Effects, Applications*. Technomic Publishing Company, Inc.: Lancaster, Pennsylvania, 1995.

[88] Macheix, J.-J.; Fleuriet, A.; Billot, J., *Fruit Phenolics*. CRC Press: Boca Raton, Florida, 1990.

[89] Hoult, J. R. S.; Payá, M., Pharmacological and biochemical actions of simple coumarins: Natural products with therapeutic potential. *Gen. Pharmacol.-Vasc. S.* **1996,** *27*, 713-722.

[90] Sharma, S. K.; Kumar, S.; Chand, K.; Kathuria, A.; Gupta, A.; Jain, R., An Update on Natural Occurrence and Biological Activity of Chromones. *Curr. Med. Chem.* **2011,** *18*, 3825-3852.

[91] Chong, J.; Poutaraud, A.; Hugueney, P., Metabolism and roles of stilbenes in plants. *Plant Sci.* **2009,** *177*, 143-155.

[92] Seigler, D. S., *Plant Secondary Metabolism*. Kluwer Academic: Dordrecht, The Netherlands, 1998.

[93] Harborne, J. B.; Williams, C. A., Advances in flavonoid research since 1992. *Phytochemistry* **2000,** *55*, 481-504.

[94] Haslam, E., *Plant Polyphenols: Vegetable Tannins Revisited*. Cambridge University Press: Cambridge, 1989.

[95] Porter, L. J., Plant Phenolics. In *Methods in Plant Biochemistry*, P.M.Dey; J.B.Harborne, Eds. Academic Press: London, 1989; Vol. 1.

[96] Khanbabaee, K.; van Ree, T., Tannins: Classification and Definition. *Nat. Prod. Rep.* **2001,** *18*, 641-649.

[97] Schofield, P.; Mbugua, D. M.; Pell, A. N., Analysis of condensed tannins: a review. *Anim. Feed Sci.Tech.* **2001,** *91*, 21-40.

[98] Manach, C.; Mazur, A.; Scalbert, A., Polyphenols and prevention of cardiovascular diseases. *Curr. Opin. Lipidol.* **2005,** *16*, 77-84.

[99] Boudet, A.-M., Evolution and current status of research in phenolic compounds. *Phytochemistry* **2007,** *68*, 2722-2735.

[100] Whiting, D. A., Natural phenolic compounds 1900-2000: a bird's eye view of a century's chemistry. *Nat. Prod. Rep.* **2001,** *18*, 583-606.

[101] Kähkönen, M. P.; Hopia, A. I.; Vuorela, H. J.; Rauha, J.-P.; Pihlaja, K.; Kujala, T. S.; Heinonen, M., Antioxidant Activity of Plant Extracts Containing Phenolic Compounds. *J. Agric. Food Chem.* **1999,** *47*, 3954-3962.

[102] Kaur, C.; Kapoor, H. C., Antioxidants in fruits and vegetables – the millennium's health. *Int. J. Food Sci. Technol.* **2001,** *36*, 703-725.

[103] RiceEvans, C. A.; Miller, J.; Paganga, G., Antioxidant properties of phenolic compounds. *Trends Plant Sci.* **1997,** *2*, 152-159.

[104] Zheng, W.; Wang, S. Y., Antioxidant Activity and Phenolic Compounds in Selected Herbs. *J. Agric. Food Chem.* **2001,** *49*, 5165-5170.

[105] Atoui, A. K.; Mansouri, A.; Boskou, G.; Kefalas, P., Tea and herbal infusions: Their antioxidant activity and phenolic profile. *Food Chem.* **2005,** *89*, 27-36.

[106] Hassan Khan, M. T.; Ather, A., Potentials of phenolic molecules of natural origin and their derivatives as anti-HIV agents. In *Biotechnology Annual Review*, El-Gewely, M. R., Ed. Elsevier: 2007; Vol. Volume 13, pp 223-264.

[107] Kim, Y. J., Antimelanogenic and antioxidant properties of gallic acid. *Biol. Pharm. Bull.* **2007,** *30*, 1052-1055.

[108] Ferreira, N.; Saraiva, M. J.; Almeida, M. R., Natural polyphenols as modulators of TTR amyloidogenesis: in vitro and in vivo evidences towards therapy. *Amyloid-J. Protein Fold. Disord.* **2012,** *19*, 39-42.

[109] Zhang, Y.; Seeram, N. P.; Lee, R.; Feng, L.; Heber, D., Isolation and Identification of Strawberry Phenolics with Antioxidant and Human Cancer Cell Antiproliferative Properties. *J. Agric. Food Chem.* **2008,** *56*, 670-675.

[110] Muthusamy, V. S.; Anand, S.; Sangeetha, K. N.; Sujatha, S.; Arun, B.; Lakshmi, B. S., Tannins present in Cichorium intybus enhance glucose uptake and inhibit adipogenesis in 3T3-L1 adipocytes through PTP1B inhibition. *Chem.-Biol. Interact.* **2008,** *174*, 69-78.

[111] Serrano, A.; Palacios, C.; Roy, G.; Cespón, C.; Villar, M. L.; Nocito, M.; González-Porqué, P., Derivatives of gallic acid induce apoptosis in tumoral cell lines and inhibit lymphocyte proliferation. *Arch. Biochem. Biophys.* **1998,** *350*, 49-54.

[112] Cueva, C.; Moreno-Arribas, M. V.; Martín-Álvarez, P. J.; Bills, G.; Vicente, M. F.; Basilio, A.; Rivas, C. L.; Requena, T.; Rodríguez, J. M.; Bartolomé, B., Antimicrobial activity of phenolic acids against commensal, probiotic and pathogenic bacteria. *Res. Microbiol.* **2010,** *161*, 372-382.

[113] Foti, M.; Piattelli, M.; Baratta, M. T.; Ruberto, G., Flavonoids, Coumarins, and Cinnamic Acids as Antioxidants in a Micellar System. Structure−Activity Relationship†. *J. Agric. Food Chem.* **1996,** *44*, 497-501.

[114] Sharma, P., Cinnamic acid derivatives: A new chapter of various pharmacological activities. *J.Chem. Pharmacol. Res.* **2011,** *3*, 403-423.

[115] Figueroa, M.; Rivero-Cruz, I.; Rivero-Cruz, B.; Bye, R.; Navarrete, A.; Mata, R., Constituents, biological activities and quality control parameters of the crude extract and essential oil from Arracacia tolucensis var. multifida. *J. Ethnopharmacol.* **2007,** *113*, 125-131.

[116] Hutter, J. A.; Salman, M.; Stavinoha, W. B.; Satsangi, N.; Williams, R. F.; Streeper, R. T.; Weintraub, S. T., Antiinflammatory C-Glucosyl Chromone from Aloe barbadensis. *J. Nat. Prod.* **1996,** *59*, 541-543.

[117] Nunthanavanit, P.; Anthony, N. G.; Johnston, B. F.; Mackay, S. P.; Ungwitayatorn, J., 3D-QSAR Studies on Chromone Derivatives as HIV-1 Protease Inhibitors: Application of Molecular Field Analysis. *Arch. Pharm.* **2008,** *341*, 357-364.

[118] Acuna, U. M.; Jancovski, N.; Kennelly, E. J., Polyisoprenylated Benzophenones from Clusiaceae: Potential Drugs and Lead Compounds. *Curr. Top. Med. Chem.* **2009,** *9*, 1560-1580.

[119] Jiang, D. J.; Dai, Z.; Li, Y. J., Pharmacological effects of xanthones as cardiovascular protective agents. *Cardiovasc. Drug Rev.* **2004,** *22*, 91-102.

[120] Roupe, K. A.; Remsberg, C. M.; Yáñez, J. A.; Davies, N. M., Pharmacometrics of stilbenes: seguing towards the clinic. *Curr. Clin. Pharmacol.* **2006,** *1*, 81-101.

[121] Kris-Etherton, P. M.; Hecker, K. D.; Bonanome, A.; Coval, S. M.; Binkoski, A. E.; Hilpert, K. F.; Griel, A. E.; Etherton, T. D., Bioactive compounds in foods: their role in the prevention of cardiovascular disease and cancer. *Am. J. Med.* **2002,** *113 Suppl 9B*, 71S-88S.

[122] Middleton Jr, E.; Kandaswami, C.; Theoharides, T. C., The effects of plant flavonoids on mammalian cells: Implications for inflammation, heart disease, and cancer. *Pharmacol. Rev.* **2000,** *52*, 673-751.

[123] Scalbert, A., Antimicrobial properties of tannins. *Phytochemistry* **1991,** *30*, 3875-3883.

[124] Okuda, T., Systematics and health effects of chemically distinct tannins in medicinal plants. *Phytochemistry* **2005,** *66*, 2012-2031.

[125] Bawadi, H. A.; Bansode, R. R.; Trappey Ii, A.; Truax, R. E.; Losso, J. N., Inhibition of Caco-2 colon, MCF-7 and Hs578T breast, and DU 145 prostatic cancer cell proliferation by water-soluble black bean condensed tannins. *Cancer Lett* **2005,** *218*, 153-162.

[126] Omar Mostafa, M., Phenolic Compounds in Botanical Extracts Used in Foods, Flavors, Cosmetics, and Pharmaceuticals. In *Phenolic Compounds in Food and Their Effects on Health I*, American Chemical Society: New Jersey, New Jersey, USA, 1992; Vol. 506, pp 154-168.

[127] Ou, S.; Kwok, K.-C., Ferulic acid: pharmaceutical functions, preparation and applications in foods. *J. Sci. Food Agric.* **2004,** *84*, 1261-1269.

[128] Mueller, R. L., First-generation agents: Aspirin, heparin and coumarins. *Best Pract. Res. Cl. Ha.* **2004,** *17*, 23-53.

[129] Andre, P.; Renimel, I. Protecting and regerating composition. US7718203, 2007.

[130] Naczk, M.; Shahidi, F., Extraction and analysis of phenolics in food. *J. Chromatogr. A* **2004,** *1054*, 95-111.

[131] Liazid, A.; Palma, M.; Brigui, J.; Barroso, C. G., Investigation on phenolic compounds stability during microwave-assisted extraction. *J. Chromatogr. A* **2007,** *1140*, 29-34.

[132] Palma, M.; Pineiro, Z.; Barroso, C. G., Stability of phenolic compounds during extraction with superheated solvents. *J. Chromatogr. A* **2001,** *921*, 169-174.

[133] Trela, B. C.; Waterhouse, A. L., Resveratrol: Isomeric molar absorptivities and stability. *J. Agric. Food Chem.* **1996,** *44*, 1253-1257.

[134] Moure, A.; Cruz, J. M.; Franco, D.; Domínguez, J. M.; Sineiro, J.; Domínguez, H.; José Núñez, M. a.; Parajó, J. C., Natural antioxidants from residual sources. *Food Chem.* **2001,** *72*, 145-171.

[135] Pinelo, M.; Fabbro, P. D.; Manzocco, L.; Nuñez, M. J.; Nicoli, M. C., Optimization of continuous phenol extraction from *Vitis vinifera* byproducts. *Food Chem.* **2005,** *92*, 109-117.

[136] Qu, W. J.; Pan, Z. L.; Ma, H. L., Extraction modeling and activities of antioxidants from pomegranate marc. *J. Food Eng.* **2010,** *99*, 16-23.

[137] Ju, Z. Y.; Howard, L. R., Effects of solvent and temperature on pressurized liquid extraction of anthocyanins and total phenolics from dried red grape skin. *J. Agric. Food Chem.* **2003,** *51*, 5207-5213.

[138] Proestos, C.; Komaltis, M., Application of microwave-assisted extraction to the fast extraction of plant phenolic compounds. *LWT-Food Sci. Technol.* **2008,** *41*, 652-659.

[139] Luthria, D. L.; Biswas, R.; Natarajan, S., Comparison of extraction solvents and techniques used for the assay of isoflavones from soybean. *Food Chem.* **2007,** *105*, 325-333.

[140] Vatai, T.; Skerget, M.; Knez, Z., Extraction of phenolic compounds from elder berry and different grape marc varieties using organic solvents and/or supercritical carbon dioxide. *J. Food Eng.* **2009,** *90*, 246-254.

[141] Rodriguez-Arcos, R. C.; Smith, A. C.; Waldron, K. W., Effect of storage on wall-bound phenolics in green asparagus. *J. Agric. Food Chem.* **2002,** *50*, 3197-3203.

[142] Stalikas, C. D., Extraction, separation, and detection methods for phenolic acids and flavonoids. *J. Sep. Sci.* **2007,** *30*, 3268-3295.

[143] Tang, B.; Bi, W.; Tian, M.; Row, K. H., Application of ionic liquid for extraction and separation of bioactive compounds from plants. *J. Chromatogr. B* **2012,** *904*, 1-21.

[144] Garcia-Salas, P.; Morales-Soto, A.; Segura-Carretero, A.; Fernandez-Gutierrez, A., Phenolic-Compound-Extraction Systems for Fruit and Vegetable Samples. *Molecules* **2010,** *15*, 8813-8826.

[145] Ballard, T. S.; Mallikarjunan, P.; Zhou, K.; O'Keefe, S. F., Optimizing the Extraction of Phenolic Antioxidants from Peanut Skins Using Response Surface Methodology. *J. Agric. Food Chem.* **2009,** *57*, 3064-3072.

[146] Mussatto, S. I.; Ballesteros, L. F.; Martins, S.; Teixeira, J. A., Extraction of antioxidant phenolic compounds from spent coffee grounds. *Sep. Purif. Technol.* **2011,** *83*, 173-179.

[147] Naczk, M.; Shahidi, F., Phenolics in cereals, fruits and vegetables: Occurrence, extraction and analysis. *J. Pharmaceut. Biomed.* **2006,** *41*, 1523-1542.

[148] Fernandes, A.; Fernandes, I.; Cruz, L.; Mateus, N.; Cabral, M.; de Freitas, V., Antioxidant and Biological Properties of Bioactive Phenolic Compounds from *Quercus suber* L. *J. Agric. Food Chem.* **2009,** *57*, 11154-11160.

[149] Tomsone, L.; Kruma, Z.; Galoburda, R., Comparison of different solvents and extraction methods for isolation of phenolic compounds from horseradish roots (*Armoracia rusticana*). *World Acad. Sci. Eng. Technol.* **2012,** *64*, 903-908.

[150] Wang, L.; Weller, C. L., Recent advances in extraction of nutraceuticals from plants. *Trends Food Sci. Tech.* **2006,** *17*, 300-312.

[151] Aspé, E.; Fernández, K., The effect of different extraction techniques on extraction yield, total phenolic, and anti-radical capacity of extracts from Pinus radiata Bark. *Ind. Crop. Prod.* **2011,** *34*, 838-844.

[152] Annegowda, H. V.; Mordi, M. N.; Ramanathan, S.; Hamdan, M. R.; Mansor, S. M., Effect of Extraction Techniques on Phenolic Content, Antioxidant and Antimicrobial Activity of Bauhinia purpurea: HPTLC Determination of Antioxidants. *Food Anal. Meth.* **2012,** *5*, 226-233.

[153] Budrat, P.; Shotipruk, A., Extraction of phenolic compounds from fruits of bitter melon (*Momordica charantia*) with subcritical water extraction and antioxidant activities of these extracts. *Chiang Mai J. Sci.* **2008,** *38*, 123-130.

[154] Domingues, R. M. A.; Patinha, D. J. S.; Sousa, G. D. A.; Villaverde, J. J.; Silva, C. M.; Freire, C. S. R.; Silvestre, A. J. D.; Neto, C. P., *Eucalyptus* biomass residues from agro-forest and pulping industries as sources of high-value triterpenic compounds. *Cell Chem. Technol.* **2011,** *45*, 475-481.

[155] Richter, B. E.; Jones, B. A.; Ezzell, J. L.; Porter, N. L.; Avdalovic, N.; Pohl, C., Accelerated solvent extraction: A technique for sample preparation. *Anal. Chem.* **1996,** *68*, 1033-1039.

[156] Ibanez, E.; Herrero, M.; Martin-Alvarez, P. J.; Senorans, F. J.; Reglero, G.; Cifuentes, A., Accelerated solvent extraction: A new procedure to obtain functional ingredients from natural sources. In *Modern Extraction Techniques: Food and Agricultural Samples*, Turner, C., Ed. 2006; Vol. 926, pp 65-78.

[157] Esclapez, M. D.; Garcia-Perez, J. V.; Mulet, A.; Carcel, J. A., Ultrasound-Assisted Extraction of Natural Products. *Food Eng. Rev.* **2011,** *3*, 108-120.

[158] Chemat, F.; Zill e, H.; Khan, M. K., Applications of ultrasound in food technology: Processing, preservation and extraction. *Ultrason. Sonochem.* **2011,** *18*, 813-835.

[159] Luque-García, J. L.; Luque de Castro, M. D., Ultrasound: a powerful tool for leaching. *Trac-Trend Anal. Chem.* **2003,** *22*, 41-47.

[160] Kaufmann, B.; Christen, P., Recent extraction techniques for natural products: Microwave-assisted extraction and pressurised solvent extraction. *Phytochem. Analysis* **2002,** *13*, 105-113.

[161] Du, F.-Y.; Xiao, X.-H.; Luo, X.-J.; Li, G.-K., Application of ionic liquids in the microwave-assisted extraction of polyphenolic compounds from medicinal plants. *Talanta* **2009,** *78*, 1177-1184.

[162] Rodriguez-Rojo, S.; Visentin, A.; Maestri, D.; Cocero, M. J., Assisted extraction of rosemary antioxidants with green solvents. *J. Food Eng.* **2012,** *109*, 98-103.

[163] Demirbas, A., Supercritical fluid extraction and chemicals from biomass with supercritical fluids. *Energ. Convers. Manage.* **2001,** *42*, 279-294.

[164] Reverchon, E.; De Marco, I., Supercritical fluid extraction and fractionation of natural matter. *J. Supercrit. Fluid* **2006,** *38*, 146-166.

[165] Díaz-Reinoso, B.; Moure, A.; Domínguez, H.; Parajó, J. C., Supercritical CO_2 extraction and purification of compounds with antioxidant activity. *J. Agric. Food Chem.* **2006,** *54*, 2441-2469.

[166] Lang, Q.; Wai, C. M., Supercritical fluid extraction in herbal and natural product studies - a practical review. *Talanta* **2001,** *53*, 771-782.

[167] Chen, H.; Zuo, Y. G.; Deng, Y. W., Separation and determination of flavonoids and other phenolic compounds in cranberry juice by high-performance liquid chromatography. *J. Chromatogr. A* **2001,** *913*, 387-395.

[168] Robbins, R. J., Phenolic Acids in Foods: An Overview of Analytical Methodology. *J. Agric. Food Chem.* **2003,** *51*, 2866-2887.

[169] Folin, O.; Denis, W., On phosphotungstic-phosphomolybdic compounds as colour reagents. *J. Biol. Chem.* **1912,** *12*, 239-243.

[170] Folin, O.; Ciocalteu, V., On tyrosine and tryptophan determinations in proteins. *J. Biol. Chem.* **1927,** *27*, 627-650.

[171] Slimestad, R.; Vangdal, E.; Brede, C., Analysis of Phenolic Compounds in Six Norwegian Plum Cultivars (Prunus domestica L.). *J. Agric. Food Chem.* **2009,** *57*, 11370-11375.

[172] Price, M. L.; Butler, L. G., Rapid visual estimation and spectrophotometric determination of tannin content of sorghum grain. *J. Agric. Food Chem.* **1977,** *25*, 1268-1273.

[173] Folin, O.; Denis, W., Tyrosine in proteins as determined by a new colorimetric method. *J. Biol. Chem.* **1912,** *12*, 245-251.

[174] Graham, H. D., Stabilization of the Prussian blue color in the determination of polyphenols. *J. Agric. Food Chem.* **1992,** *40*, 801-805.

[175] Singleton, V. L.; Rossi, J. A., Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enol. Viticult.* **1965,** *16*, 144-158.

[176] Vinson, J. A.; Su, X.; Zubik, L.; Bose, P., Phenol Antioxidant Quantity and Quality in Foods: Fruits. *J. Agric. Food Chem.* **2001,** *49*, 5315-5321.

[177] Maranz, S.; Wiesman, Z.; Garti, N., Phenolic Constituents of Shea (*Vitellaria paradoxa*) Kernels. *J. Agric. Food Chem.* **2003,** *51*, 6268-6273.

[178] Velioglu, Y. S.; Mazza, G.; Gao, L.; Oomah, B. D., Antioxidant Activity and Total Phenolics in Selected Fruits, Vegetables, and Grain Products. *J. Agric. Food Chem.* **1998,** *46*, 4113-4117.

[179] Sellappan, S.; Akoh, C. C.; Krewer, G., Phenolic Compounds and Antioxidant Capacity of Georgia-Grown Blueberries and Blackberries. *J. Agric. Food Chem.* **2002,** *50*, 2432-2438.

[180] Prior, R. L.; Wu, X. L.; Schaich, K., Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *J. Agric. Food Chem.* **2005,** *53*, 4290-4302.

[181] Vermerris, W.; Nicholson, R., Isolation and Identification of Phenolic Compounds. In *Phenolic Compound Biochemistry*, Springer Netherlands: 2006; pp 151-196.

[182] Škerget, M.; Kotnik, P.; Hadolin, M.; Hraš, A. R.; Simonič, M.; Knez, Ž., Phenols, proanthocyanidins, flavones and flavonols in some plant materials and their antioxidant activities. *Food Chem.* **2005,** *89*, 191-198.

[183] Petridis, A.; Therios, I.; Samouris, G.; Koundouras, S.; Giannakoula, A., Effect of water deficit on leaf phenolic composition, gas exchange, oxidative damage and antioxidant activity of four Greek olive (Olea europaea L.) cultivars. *Plant Physiol. Bioch.* **2012,** *60*, 1-11.

[184] Petridis, A.; Therios, I.; Samouris, G., Genotypic Variation of Total Phenol and Oleuropein Concentration and Antioxidant Activity of 11 Greek Olive Cultivars (Olea europaea L.). *Hortscience* **2012,** *47*, 339-342.

[185] Scalbert, A.; Monties, B.; Janin, G., Tannins in wood: comparison of different estimation methods. *J. Agric. Food Chem.* **1989,** *37*, 1324-1329.

[186] Vazquez, G.; Gonzalez-Alvarez, J.; Santos, J.; Freire, M. S.; Antorrena, G., Evaluation of potential applications for chestnut (*Castanea sativa*) shell and eucalyptus (*Eucalyptus globulus*) bark extracts. *Ind. Crop. Prod.* **2009,** *29*, 364-370.

[187] Mota, I.; Rodrigues Pinto, P. C.; Novo, C.; Sousa, G.; Guerreiro, O.; Guerra, A. R.; Duarte, M. F.; Rodrigues, A. E., Extraction of polyphenolic compounds from *Eucalyptus globulus* cark: Process optimization and screening for biological activity. *Ind. Eng.Chem. Res.* **2012,** *51*, 6991-7000.

[188] Haslam, E., Galloyl esters in the Aceraceae. *Phytochemistry* **1965,** *4*, 495-498.

[189] Bate-Smith, E. C., Astringency of leaves.1.Astringent tannins of Acer species. *Phytochemistry* **1977,** *16*, 1421-1426.

[190] Mueller-Harvey, I., Analysis of hydrolysable tannins. *Anim. Feed Sci. Technol.* **2001,** *91*, 3-20.

[191] Hartzfeld, P. W.; Forkner, R.; Hunter, M. D.; Hagerman, A. E., Determination of hydrolyzable tannins (gallotannins and ellagitannins) after reaction with potassium iodate. *J. Agric. Food Chem.* **2002,** *50*, 1785-1790.

[192] Willis, R. B.; Allen, P. R., Improved method for measuring hydrolyzable tannins using potassium iodate. *Analyst* **1998,** *123*, 435-439.

[193] Inoue, K. H.; Hagerman, A. E., Determination of gallotannin with rhodanine. *Anal. Biochem.* **1988,** *169*, 363-369.

[194] Wilson, T. C.; Hagerman, A. E., Quantitative determination of ellagic acid. *J. Agric. Food Chem.* **1990,** *38*, 1678-1683.

[195] Salminen, J. P.; Roslin, T.; Karonen, M.; Sinkkonen, J.; Pihlaja, K.; Pulkkinen, P., Seasonal variation in the content of hydrolyzable tannins, flavonoid glycosides, and proanthocyanidins in oak leaves. *J. Chem. Ecol.* **2004,** *30*, 1693-1711.

[196] Elfalleh, W.; Tlili, N.; Nasri, N.; Yahia, Y.; Hannachi, H.; Chaira, N.; Ying, M.; Ferchichi, A., Antioxidant Capacities of Phenolic Compounds and Tocopherols from Tunisian Pomegranate (Punica granatum) Fruits. *J. Food Sci.* **2011,** *76*, C707-C713.

[197] Berardini, N.; Carle, R.; Schieber, A., Characterization of gallotannins and benzophenone derivatives from mango (Mangifera indica L. cv. 'Tommy Atkins') peels, pulp and kernels by high-performance liquid chromatography electrospray ionization mass spectrometry. *Rapid Commun. Mass Spectrom.* **2004,** *18*, 2208-2216.

[198] Salminen, J.-P., Effects of Sample Drying and Storage, and Choice of Extraction Solvent and Analysis Method on the Yield of Birch Leaf Hydrolyzable Tannins. *J. Chem. Ecol.* **2003,** *29*, 1289-1305.

[199] Hagerman, A. E.; Butler, L. G., Choosing appropriate methods and standards for assaying tannin. *J. Chem. Ecol.* **1989,** *15*, 1795-1810.

[200] Porter, L. J.; Hrstich, L. N.; Chan, B. G., The conversion of procyanidins and prodelphinidins to cyanidin and delphinidin. *Phytochemistry* **1985,** *25*, 223-230.

[201] Naczk, M.; Nichols, T.; Pink, D.; Sosulski, F., Condensed tannins in canola hulls. *J. Agric. Food Chem.* **1994,** *42*, 2196-2200.

[202] Amarowicz, R.; Troszynska, A.; Barylko-Pikielna, N.; Shahidi, F., Polyphenolics extracts from legume seeds: Correlations between total antioxidant activity, total phenolics content, tannins content and astringency. *J. Food Lipids* **2004,** *11*, 278-286.

[203] Yazaki, Y.; Hillis, W. E., MOLECULAR-SIZE DISTRIBUTION OF RADIATA PINE BARK EXTRACTS AND ITS EFFECT ON PROPERTIES. *Holzforschung* **1980,** *34*, 125-130.

[204] Stiasny, E., Die Einwirkung von Formaldehyde auf Berbstoffe. *Der Gerber* **1905,** *740*, 186-188.

[205] Tahir, P. M.; Musgrave, O. C.; Ashaari, Z., Determination of polyphenolic content of bark extracts for wood adhesives. *Holzforschung* **2002,** *56*, 267-272.

[206] Schimleck, L. R.; Yazaki, Y., Analysis of Pinus radiata D. Don bark by near infrared spectroscopy. *Holzforschung* **2003,** *57*, 520-526.

[207] Hafizoglu, H.; Usta, M.; Bilgin, O., Wood and bark composition of *Picea orientalis* (L) Link. *Holzforschung* **1997,** *51*, 114-118.

[208] Tomas-Barberan, F. A., Capillary Electrophoresis - A new technique in the analysis of plant secondary metabolites. *Phytochem. Analysis* **1995,** *6*, 177-192.

[209] Nonier, M. F.; Absalon, C.; Vivas, N.; Vivas de Gaulejac, N., Application of off-line size-exclusion chromatographic fractionation–matrix assisted laser desorption ionization time of flight mass spectrometry for proanthocyanidin characterization. *J. Chromatogr. A* **2004,** *1033*, 291-297.

[210] Sajewicz, M.; Staszek, D.; Waksmundzka-Hajnos, M.; Kowalska, T., Comparison of TLC and HPLC fingerprints of phenolic acids and flavonoids fractions derived from selected sage (Salvia) species. *J. Liq. Chromatogr. Relat. Technol.* **2012,** *35*, 1388-1403.

[211] Cadahía, E.; Conde, E.; García-Vallejo, M. C.; de Simón, B. F., Gel permeation chromatographic study of the molecular weight distribution of tannins in the wood, bark and leaves of *Eucalyptus* spp. *Chromatographia* **1996,** *42*, 95-100.

[212] Liggins, J.; Bluck, L. J. C.; Coward, W. A.; Bingham, S. A., Extraction and quantification of daidzein and genistein in food. *Anal. Biochem.* **1998,** *264*, 1-7.

[213] Bankova, V.; Dyulgerov, A.; Popov, S.; Evstatieva, L.; Kuleva, L.; Pureb, O.; Zamjansan, Z., Propolis produced in Bulgaria and Mongolia - Phenolic compounds and plant-origin. *Apidologie* **1992,** *23*, 79-85.

[214] Schulz, J. M.; Herrmann, K., Analysis of hydroxybenzoic and hydroxycinnamic acids in plant material : II. Determination by gas-liquid chromatography. *J. Chromatogr. A* **1980,** *195*, 95-104.

[215] Yazaki, K.; Okuda, T., Ellagitannin formation in callus cultures of Heterocentron roseum. *Phytochemistry* **1990,** *29*, 1127-1130.

[216] Conde, E.; Cadahía, E.; García-Vallejo, M. C., Optimization of TLC for research on the flavonoids in wood and bark of species of the genus *Eucalyptus*-L'Héritier. *Chromatographia* **1992,** *33*, 418-426.

[217] Engelhardt, H.; Gutnikov, G., *High performance liquid chromatography*. Springer-Verlag: New York, New York, U.S.A., 1979.

[218] Brown, P.; Hartwick, R. A., *High performance liquid chromatography*. John Wiley and Sons, Inc: New York, New York, 1988.

[219] Sakakibara, H.; Honda, Y.; Nakagawa, S.; Ashida, H.; Kanazawa, K., Simultaneous determination of all polyphenols in vegetables, fruits, and teas. *J. Agric. Food Chem.* **2003,** *51*, 571-581.

[220] Van Sumere, C.; Fache, P.; Vande Casteele, K.; De Cooman, L.; Everaert, E.; De Loose, R.; Hutsebaut, W., Improved extraction and reversed phase-high performance liquid chromatographic separation of flavonoids and the identification of Rosa cultivars. *Phytochem. Analysis* **1993,** *4*, 279-292.

[221] Monagas, M.; Suarez, R.; Gomez-Cordoves, C.; Bartolome, B., Simultaneous determination of nonanthocyanin phenolic compounds in red wines by HPLC-DAD/ESI-MS. *Am. J. Enol. Viticult.* **2005,** *56*, 139-147.

[222] Guyot, S.; Doco, T.; Souquet, J.-M.; Moutounet, M.; Drilleau, J.-F., Characterization of highly polymerized procyanidins in cider apple (*Malus sylvestris var. kermerrien*) skin and pulp. *Phytochemistry* **1997,** *44*, 351-357.

[223] Lazarus, S. A.; Adamson, G. E.; Hammerstone, J. F.; Schmitz, H. H., High-Performance Liquid Chromatography/Mass Spectrometry Analysis of Proanthocyanidins in Foods and Beverages. *J. Agric. Food Chem.* **1999,** *47*, 3693-3701.

[224] Kalili, K. M.; de Villiers, A., Recent developments in the HPLC separation of phenolic compounds. *J. Sep. Sci.* **2011,** *34*, 854-876.

[225] Kumar, N.; Bhandari, P.; Singh, B.; Bari, S. S., Antioxidant activity and ultra-performance LC-electrospray ionization-quadrupole time-of-flight mass spectrometry for phenolics-based fingerprinting of Rose species: Rosa damascena, Rosa bourboniana and Rosa brunonii. *Food Chem. Toxicol.* **2009,** *47*, 361-367.

[226] Klejdus, B.; Vacek, J.; Lojkova, L.; Benesova, L.; Kuban, V., Ultrahigh-pressure liquid chromatography of isoflavones and phenolic acids on different stationary phases. *J. Chromatogr. A* **2008,** *1195*, 52-59.

[227] Oleszek, W.; Stochmal, A.; Janda, B., Concentration of Isoflavones and Other Phenolics in the Aerial Parts of Trifolium Species. *J. Agric. Food Chem.* **2007,** *55*, 8095-8100.

[228] Rostagno, M. A.; Manchón, N.; D'Arrigo, M.; Guillamón, E.; Villares, A.; García-Lafuente, A.; Ramos, A.; Martínez, J. A., Fast and simultaneous determination of phenolic compounds and caffeine in teas, mate, instant coffee, soft drink and energetic drink by high-performance liquid chromatography using a fused-core column. *Anal. Chim. Acta* **2011,** *685*, 204-211.

[229] Padda, M. S.; Picha, D. H., Methodology optimization for quantification of total phenolics and individual phenolic acids in sweetpotato (Ipomoea batatas L.) roots. *J. Food Sci.* **2007,** *72*, C412-C416.

[230] López, M.; Martínez, F.; Del Valle, C.; Orte, C.; Miró, M., Analysis of phenolic constituents of biological interest in red wines by high-performance liquid chromatography. *J. Chromatogr. A* **2001,** *922*, 359-363.

[231] Gennaro, L.; Leonardi, C.; Esposito, F.; Salucci, M.; Maiani, G.; Quaglia, G.; Fogliano, V., Flavonoid and carbohydrate contents in tropea red onions: Effects of homelike peeling and storage. *J. Agric. Food Chem.* **2002,** *50*, 1904-1910.

[232] Tsao, R.; Deng, Z. Y., Separation procedures for naturally occurring antioxidant phytochemicals. *J. Chromatogr. B* **2004,** *812*, 85-99.

[233] Larson, J. R.; Tingstad, J. E.; Swadesh, J. K., Introduction. In *HPLC Practical and Industrial Applications*, 2nd ed.; Swadesh, J. K., Ed. CRC Press: Boca Raton, Florida, U.S.A., 2001; pp 1-71.

[234] Lores, M.; Garcia, C. M.; Cela, R., High-Performance Liquid-Chromatography of phenolic aldehydes with highly selective flurometric detection by means of postcolumn photochemical derivatization. *J. Chromatogr. A* **1994,** *683*, 31-44.

[235] Snyder, L. R.; Kirkland, J. J.; Dolan, J. W., *Introduction to Modern Liquid Chromatography*. John Wiley & Sons, Inc.: New Jersey, U.S.A., 2010.

[236] Watson, J. T., *Introduction to Mass Spectrometry*. 2nd ed.; Raven Press: New York, U.S.A, 1985.

[237] Ng, L. K.; Lafontaine, P.; Vanier, M., Characterization of ciggarette tobacco by direct electrospray ionization-ion trap mass spectrometry (ESI-ITMS) analyis of the aqueous extract - A novel and simple approach. *J. Agric. Food Chem.* **2004,** *52*, 7251-7257.

[238] Dass, C., *Fundamentals of Contemporary Mass Spectrometry*. John Wiley & Sons, Inc.: New Jersey, U.S.A., 2007.

[239] Abad-Garcia, B.; Berrueta, L. A.; Garmon-Lobato, S.; Gallo, B.; Vicente, F., A general analytical strategy for the characterization of phenolic compounds in fruit juices by high-performance liquid chromatography with diode array detection coupled to electrospray ionization and triple quadrupole mass spectrometry. *J. Chromatogr. A* **2009,** *1216*, 5398-5415.

[240] Alonso-Salces, R. M.; Guillou, C.; Berrueta, L. A., Liquid chromatography coupled with ultraviolet absorbance detection, electrospray ionization, collision-induced dissociation and tandem mass spectrometry on a triple quadrupole for the on-line characterization of polyphenols and methylxanthines in green coffee beans. *Rapid Commun. Mass Spectrom.* **2009,** *23*, 363-383.

[241] Ardrey, B., *Liquid Chromatography-Mass Spectrometry: An Introduction*. John Wiley & Sons, Inc.: Chichester, England, 2003.

[242] Wilson, I. D.; Davis, P.; Ruane, R. J., Supercritical fluid chromatography and extraction of pharmaceuticals. In *Applications of Supercritical Fluids in Industrial Analysis*, Dean, J. R., Ed. Springer Netherlands: 1993; pp 74-103.

[243] Bernal, J. L.; Nozal, M. J.; Toribio, L.; Serna, M. L.; Borrull, F.; Marce, R. M.; Pocurull, E., Determination of phenolic compounds in water samples by on-line solid-phase extraction - Supercritical-fluid chromatography with diode-array detection. *Chromatographia* **1997,** *46*, 295-300.

[244] Ramírez, P.; Santoyo, S.; García-Risco, M. R.; Señoráns, F. J.; Ibáñez, E.; Reglero, G., Use of specially designed columns for antioxidants and antimicrobials enrichment by preparative supercritical fluid chromatography. *J. Chromatogr. A* **2007,** *1143*, 234-242.

[245] Kamangerpour, A.; Ashraf-Khorassani, M.; Taylor, L. T.; McNair, H. M.; Chorida, L., Supercritical fluid chromatography of polyphenolic compounds in grape seed extract. *Chromatographia* **2002,** *55*, 417-421.

[246] Ito, N.; Hirose, M.; Fukushima, S.; Tsuda, H.; Shirai, T.; Tatematsu, M., Studies on antioxidants: Their carcinogenic and modifying effects on chemical carcinogenesis. *Food Chem. Toxicol.* **1986,** *24*, 1071-1082.

[247] Whysner, J.; Wang, C. X.; Zang, E.; Iatropoulos, M. J.; Williams, G. M., Dose response of promotion by butylated hydroxyanisole in chemically initiated tumours of the rat forestomach. *Food Chem. Toxicol.* **1994,** *32*, 215-222.

[248] Halliwell, B.; Gutteridge, J. M., The definition and measurement of antioxidants in biological systems. *Free radical bio. med.* **1995,** *18*, 125-126.

[249] Frankel, E. N.; Meyer, A. S., The problems of using one-dimensional methods to evaluate multifunctional food and biological antioxidants. *J. Sci. Food Agric.* **2000,** *80*, 1925-1941.

[250] Rice-Evans, C. A.; Miller, N. J.; Paganga, G., Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Bio. Med.* **1996,** *20*, 933-956.

[251] Pietta, P.-G., Flavonoids as Antioxidants. *J. Nat. Prod.* **2000,** *63*, 1035-1042.

[252] Yokozawa, T.; Chen, C. P.; Dong, E.; Tanaka, T.; Nonaka, G.-I.; Nishioka, I., Study on the Inhibitory Effect of Tannins and Flavonoids against the 1,1-Diphenyl-2-picrylhydrazyl Radical. *Biochem. Pharmacol.* **1998,** *56*, 213-222.

[253] Huang, D. J.; Ou, B. X.; Prior, R. L., The chemistry behind antioxidant capacity assays. *J. Agric. Food Chem.* **2005,** *53*, 1841-1856.

[254] Cao, G.; Alessio, H. M.; Cutler, R. G., Oxygen-radical absorbance capacity assay for antioxidants. *Free Radical Bio. Med.* **1993,** *14*, 303-311.

[255] Siger, A.; Czubinski, J.; Kachlicki, P.; Dwiecki, K.; Lampart-Szczapa, E.; Nogala-Kalucka, M., Antioxidant activity and phenolic content in three lupin species. *J. Food Compos. Anal.* **2012,** *25*, 190-197.

[256] Moon, J.-K.; Shibamoto, T., Antioxidant Assays for Plant and Food Components. *J. Agric. Food Chem.* **2009,** *57*, 1655-1666.

[257] Moyo, M.; Ndhlala, A. R.; Finnie, J. F.; Van Staden, J., Phenolic composition, antioxidant and acetylcholinesterase inhibitory activities of Sclerocarya birrea and Harpephyllum caffrum (Anacardiaceae) extracts. *Food Chem.* **2010,** *123*, 69-76.

[258] Ozturk, M.; Aydogmus-Ozturk, F.; Duru, M. E.; Topcu, G., Antioxidant activity of stem and root extracts of Rhubarb (*Rheum ribes*): An edible medicinal plant. *Food Chem.* **2007,** *103*, 623-630.

[259] Benzie, I. F. F., An automated, specific, spectrophotometric method for measuring ascorbic acid in plasma (EFTSA). *Clinical Biochem.* **1996,** *29*, 111-116.

[260] Gorinstein, S.; Park, Y.-S.; Heo, B.-G.; Namiesnik, J.; Leontowicz, H.; Leontowicz, M.; Ham, K.-S.; Cho, J.-Y.; Kang, S.-G., A comparative study of phenolic compounds and antioxidant and antiproliferative activities in frequently consumed raw vegetables. *Eur. Food Res. Technol.* **2009,** *228*, 903-911.

[261] Oxis International Antioxidant biomarkers AOP-490 assay. http://www.oxisresearch.com/pub/PDF/inserts/AOP-490.pdf (October 12, 2012)

[262] Apak, R.; Güçlü, K.; Özyürek, M.; Karademir, S. E., Novel Total Antioxidant Capacity Index for Dietary Polyphenols and Vitamins C and E, Using Their Cupric Ion Reducing Capability in the Presence of Neocuproine: CUPRAC Method. *J. Agric. Food Chem.* **2004,** *52*, 7970-7981.

[263] Miller, N. J.; Rice-Evans, C.; Davies, M. J.; Gopinathan, V.; Milner, A., A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. *Clin. Sci.* **1993,** *84*, 407-412.

[264] Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C., Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Bio. Med.* **1999,** *26*, 1231-1237.

[265] Luximon-Ramma, A.; Bahorun, T.; Soobrattee, M. A.; Aruoma, O. I., Antioxidant activities of phenolic, proanthocyanidin, and flavonoid components in extracts of *Cassia fistula*. *J. Agric. Food Chem.* **2002,** *50*, 5042-5047.

[266] Babbar, N.; Oberoi, H. S.; Uppal, D. S.; Patil, R. T., Total phenolic content and antioxidant capacity of extracts obtained from six important fruit residues. *Food Res. Int.* **2011,** *44*, 391-396.

[267] Brand-Williams, W.; Cuvelier, M. E.; Berset, C., Use of a free radical method to evaluate antioxidant activity. *LWT - Food Sci. Technol.* **1995,** *28*, 25-30.

[268] Jiménez, A.; Selga, A.; Torres, J. L.; Julià, L., Reducing Activity of Polyphenols with Stable Radicals of the TTM Series. Electron Transfer versus H-Abstraction Reactions in Flavan-3-ols. *Org. Lett.* **2004,** *6*, 4583-4586.

[269] Foti, M. C.; Daquino, C.; Geraci, C., Electron-transfer reaction of cinnamic acids and their methyl esters with the DPPH• radical in alcoholic solutions. *J. Org. Chem.* **2004,** *69*, 2309-2314.

[270] Ozcelik, B.; Lee, J. H.; Min, D. B., Effects of light, oxygen, and pH on the absorbance of 2,2-diphenyl-1-picrylhydrazyl. *J. Food Sci.* **2003,** *68*, 487-490.

[271] Scherer, R.; Godoy, H. T., Antioxidant activity index (AAI) by the 2,2-diphenyl-1-picrylhydrazyl method. *Food Chem.* **2009,** *112*, 654-658.

[272] Sharma, O. P.; Bhat, T. K., DPPH antioxidant assay revisited. *Food Chem.* **2009,** *113*, 1202-1205.

[273] Faustino, H.; Gil, N.; Baptista, C.; Duarte, A. P., Antioxidant Activity of Lignin Phenolic Compounds Extracted from Kraft and Sulphite Black Liquors. *Molecules* **2010,** *15*, 9308-9322.

[274] Zongo, C.; Savadogo, A.; Ouattara, L.; Bassole, I. H. N.; Ouattara, C. A. T.; Ouattara, A. S.; Barro, N.; Koudou, J.; Traore, A. S., Polyphenols Content, Antioxidant and Antimicrobial Activities of Ampelocissus grantii (Baker) Planch. (Vitaceae): A Medicinal Plant from Burkina Faso. *Int. J. Pharm.* **2010,** *6*, 880-887.

[275] Vasic, S. M.; Stefanovic, O. D.; Licina, B. Z.; Radojevic, I. D.; Comic, L. R., Biological activities of extracts from cultivated Granadilla *Passiflora alata*. *Excli J.* **2012,** *11*, 208-218.

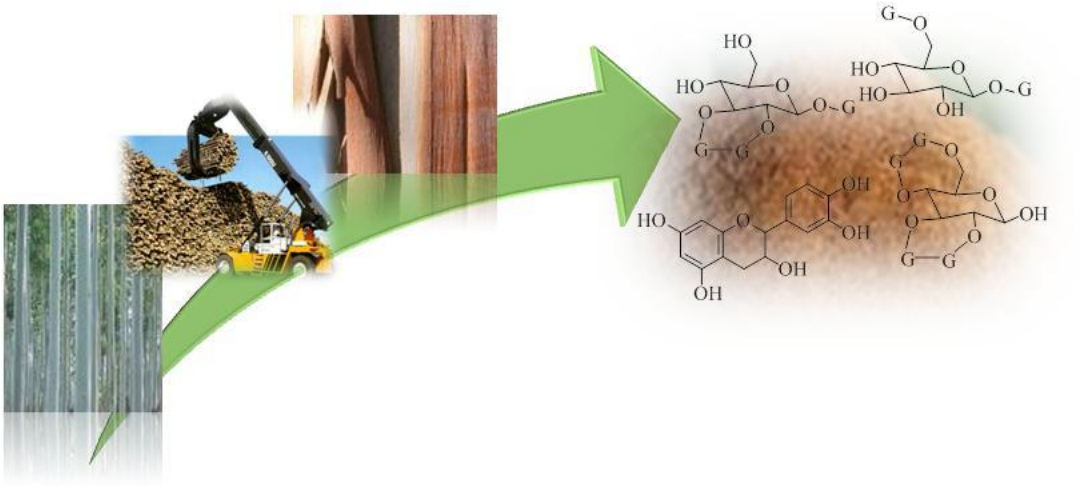
[276] El-Moein, N. M.; Mahmoud, E. A.; Shalaby, E. A., Antioxidant mechanism of active ingredients separated from *Eucalyptus globulus*. *Org.Chem. Curr. Res.* **2012,** *1*, 1-7.

Chapter 3

Eucalyptus globulus Labill. bark as a source of phenolic compounds

Part A

Characterisation of phenolic fraction of *Eucalyptus globulus* Labill. bark



Adapted from:

Santos, S.A.O., Freire, C.S.R., Domingues, M.R.M., Silvestre, A.J.D., Neto, C.P., Characterization of phenolic components in polar extracts of *Eucalyptus globulus* Labill. Bark by high-performance liquid chromatography-mass spectrometry, *Journal of Agricultural and Food Chemistry*, **2011**, 59, 9386-9393

Abstract

High-performance liquid chromatography–electrospray ionization mass spectrometry (HPLC–ESI–MS) and multi-stage mass spectrometry (MS^n) were used to investigate the phenolic constituents in methanol, water, and methanol/water extracts of *Eucalyptus globulus* Labill. bark. Twenty-nine phenolic compounds were identified, 16 of them referenced for the first time as constituents of *E. globulus* bark, namely, quinic, dihydroxyphenylacetic, and caffeic acids, bis-hexahydroxydiphenoyl (HHDP)-glucose, galloyl-bis-HHDP-glucose, galloyl-HHDP-glucose, isorhamentin-hexoside, quercetin-hexoside, methyl-ellagic acid (EA)-pentoside, myricetin-rhamnoside, isorhamnetin-rhamnoside, mearnsetin, phloridzin, mearnsetin-hexoside, luteolin, and a proanthocyanidin B-type dimer. Digalloylglucose was identified as the major compound in the methanol and methanol/water extracts, followed by isorhamnetin-rhamnoside, in the methanol extract, and by catechin, in the methanol/water extract, whereas in the water extract catechin and galloyl-HHDP-glucose were identified as the predominant components. The methanol/water extract was shown be the most efficient to isolate phenolic compounds identified in *E. globulus* bark.

3.1 Introduction

Eucalyptus globulus Labill. is one of the main wood species produced in Portugal; it ranks third in terms of Portuguese forest area (about 672,000 ha), representing about 31% of the world production of *E. globulus*, and it is the main raw material for pulp and paper production in Portugal and Spain [1]. The pulp industries generate substantial amounts of biomass residues, among which bark is the most abundant and is currently simply burned to produce energy. In the case of *E. globulus*, bark represents about 11% of the stem dry weight [2]. Thus, a pulp mill with a production capacity of 5.0×10^5 tonnes/year of bleached kraft pulp can generate around 1.0×10^5 tonnes/year of bark, showing the enormous potential for the upgrading of this biomass residue. Therefore, the detailed study of its chemical composition is a key step toward the implementation of strategies for the recovery of valuable components from this biomass residue.

Moreover, this strategy is perfectly in tune with the emerging biorefinery concept [3], which has been attracting increasing interest in recent years, from the perspective of promoting the integrated exploitation of agro-forest biomass resources in the search for new alternatives to petrochemical-derived products.

In recent years, we have demonstrated [4, 5] that the lipophilic fraction of *E. globulus* bark (and particularly its outer fraction) is quite rich in high-value triterpenic acids such as ursolic and oleanolic acids (up to 25 g/kg). However, bark is also known to be a promising source of phenolic compounds. In fact, several studies have already addressed the phenolic composition of wood and barks from several *Eucalyptus* species. Methyl and glycosyl derivatives of ellagic acid and free ellagic and gallic acids have already been reported in methanolic extracts from the bark of *E. regnans* and *E. globulus* [6]. Fechtal and Riedl [7] have also reported the presence of gallic and ellagic acid derivatives and catechin in extracts obtained after acid hydrolysis of bark from four *Eucalyptus* species. Conde [8] and Cadahía [9] and respective co-workers detected gallic and ellagic acids, vanillin, syringaldehyde, sinapaldehyde, and quercetin in methanolic extracts from *E. globulus* wood and gallic, protocatechuic, vanillic, and ellagic acids, protocatechuic aldehyde, taxifolin, eriodictyol, quercetin, and naringenin in the corresponding bark. More recently, Vázquez *et al.* [10] identified some phenolic compounds from an aqueous extract of *E. globulus* bark, including ellagic acid, galloylglucose derivatives, and flavonoids and reported also their potential as natural antioxidants. Besides this well-known property of phenolic compounds, their interest is also based in a wide variety of other valuable properties, namely, anti-inflammatory,

antithrombotic, antimicrobial, and antibacterial capacities [11], among others. The identification of phenolic compounds in vegetal matrices is a relatively complex task due to the wide variety of structures that can be found. Flavonoid glycosides are predominant forms of secondary metabolites in plants, in which the flavonoid moiety can be bound to up to five different sugar moieties, either through phenolic -OH groups in the case of *O*-glycosides or directly to carbon atoms in ring A of the flavonoid moiety in *C*-glycosides [12]. Other groups of well-known plant phenolic compounds are hydrolysable tannins, containing both galloylglucose derivatives and ellagitannins [13]. Analysis of these compounds is usually carried out by using high-performance liquid chromatography, although gas chromatography and capillary electrophoresis have also been employed [13]. All of these techniques are coupled to different detection systems, but, mostly, with mass spectrometry, which provides valuable structural information about the eluted compounds, especially when multi-stage mass spectrometry techniques are available and even when co-elution might occur. Due to the importance of phenolic compounds, as well as the interest in their identification and quantification in *E. globulus* bark, the present study reports the detailed characterisation of the phenolic fraction of *E. globulus* methanol, methanol/water, and water bark extracts, taking advantage of the use of MS/MS (obtained in a triple quadrupole) and MS^n (acquired in an ion trap mass spectrometer).

3.2 Materials and methods

3.2.1 Chemicals

Dichloromethane (99% purity), gallic acid (purity > 97.5%), quercetin (purity > 98%), and luteolin (purity > 98%) were supplied by Sigma Chemical Co (Madrid, Spain). Protocatechuic acid (purity > 97%), chlorogenic acid (purity > 95%), caffeic acid (purity > 95%), and naringenin (98% purity) were obtained from Aldrich Chemical Co. (Madrid, Spain). HPLC-grade methanol, water, and acetonitrile were supplied from Fisher Scientific Chemicals (Loures, Portugal). Formic acid (purity > 98%), methanol (purity > 99.8%), catechin (purity > 96%), and ellagic acid (96% purity) were purchased from Fluka Chemie (Madrid, Spain). Solvents were filtered using a Solvent Filtration Apparatus 58061 from Supelco (Bellefonte, PA).

3.2.2 Raw material

E. globulus bark samples were taken from 16-year-old *E. globulus* trees randomly harvested from a clone plantation cultivated by RAIZ-Forest and paper Research

Institute in Eixo (40º37' 13.56'' N, 8º34' 08.43'' W), region of Aveiro, Portugal. *E. globulus* bark samples were air-dried, until a constant weight was achieved, and ground to granulometry lower than 2 mm prior to extraction.

3.2.3 Phenolic compounds extraction

About 45 g of dried bark was submitted to a soxhlet extraction with dichloromethane for 6 h, to remove the lipophilic components [4, 5]. Then, the solid bark residue was divided in two fractions (I and II), which followed two distinct extraction pathways. Fraction I was submitted to methanol (MeOH) extraction (m/v 1:100) for 24 h, under constant stirring, followed by an extraction with water (m/v 1:100) for 24 h, both at room temperature. Methanol was then removed from the liquid extracts by low-pressure evaporation, and the residues/aqueous solutions were freeze-dried. Fraction II was suspended (m/v 1:100) in a methanol/water (MeOH/H_2O) mixture, 50:50 (v/v), at room temperature, for 24 h under constant stirring. The suspension was then filtered, MeOH removed by low-pressure evaporation, and the extract freeze-dried.

3.2.4 Total phenolic content

The total phenolic content (TPC) of the extracts was determined by the Folin-Ciocalteu method [14, 15]. Two and a half millilitres of Folin-Ciocalteu reagent, previously diluted with water (1:10, v/v), and 2 mL of aqueous sodium carbonate (75 g L^{-1}) were added to accurately weighed aliquots of the extracts dissolved in 0.5 mL of water for the H_2O extract and in methanol for the others, corresponding to concentration ranges between 35 and 500 µg of extract mL^{-1}. Each mixture was kept for 5 min at 50 ºC and, after cooling, the absorbance was measured at 760 nm, using a UV-vis V-530 spectrophotometer (Jasco, Tokyo, Japan). The TPC was calculated as gallic acid equivalents from the calibration curve of gallic acid standard solutions (1.5 - 60.0 µg mL^{-1}) and expressed as mg of gallic acid equivalent (GAE) g^{-1} of dry extract. The analyses were carried using three aliquots of each extract, measured in triplicate, and the average value was calculated in each case.

3.2.5 HPLC–UV procedure

HPLC-UV analysis were carried in a Hewlett-Packard (HP) 1050 liquid chromatograph (Agilent Technologies, Waldbronn, Germany) equipped with a Rheodyne injector with a 10 µL loop, a quaternary pumping system, and a UV detector. The column used was a Discovery C-18 (15 cm x 2.1 mm x 5 µm) supplied by Supelco (Agilent Technologies). The separation of the compounds from *E. globulus* bark extracts was carried out at

room temperature with a gradient elution program at a flow rate of 0.2 mL min^{-1}. The mobile phases consisted of water/acetonitrile (90:10, v/v) (A) and acetonitrile (B), both with 0.1% of formic acid. The following linear gradient was applied: 0-3 min, 0% B; 3-10 min, 0-10% B; 10-30 min, 10-20% B; 30-35 min, 20-25% B; 35-50 min, 25-50% B; 50-60 min, 50-0% B; followed by re-equilibration of the column for 10 min before the next run. The injection volume in the HPLC system was 25 µL, and UV-vis detection was performed at 280 and 340 nm. Before the injection in the HPLC, each extract was dissolved in the same solvent used for extraction (HPLC grade), to obtain a final concentration of about 10 mg mL^{-1}, and, then, filtered through a 0.2 µm PTFE syringe filter.

3.2.6 ESI–QqQ–MS analysis

The HPLC system was coupled to a Micromass spectrometer (Manchester, U.K.), operating in negative mode, equipped with an electrospray source and a triple-quadrupole (QqQ–MS) analyser. The cone and capillary voltages were set at -30.0 V and -2.6 kV, respectively. The source temperature was 143 ºC, and the desolvation temperature was 350 ºC. MS/MS spectra were obtained using argon as collision gas, with the collision energy set between 10 and 45 V. Detection was carried out considering a mass range of *m/z* 50-1000, with a scan duration of 0.5 s. Data acquisition was performed using the MassLynx data system (Waters, Milford, MA).

3.2.7 ESI–IT–MS–MS analysis

To gather additional MS information about several chromatographic peaks, these were manually collected following the conditions discussed above. The ensuing HPLC fractions were dissolved in methanol and directly injected into a Linear Ion Trap LXQ (ThermoFinnigan, San Jose, CA), also equipped with an ESI interface by means of a syringe pump, at flow rate of 8 µL min^{-1}. Optimal ESI conditions were as follows: nitrogen sheath gas, 30 psi; spray voltage, 4.7 kV; capillary temperature, 275 ºC; capillary voltage, -7.0 V' and tube lens voltage, -71.8 V. CID-MS/MS and MS^n experiments were performed on mass-selected precursor ions using standard isolation and excitation configurations. The collision energy used was in the range of 15 - 40 (arbitrary units). Data acquisition was carried out with the Xcalibur data system (ThermoFinnigan).

3.2.8 HPLC–UV quantification

HPLC–UV calibration curves were obtained by injection of gallic acid, protocatechuic acid, catechin, chlorogenic acid, caffeic acid, ellagic acid, quercetin, and naringenin standard solutions in MeOH, with five different concentrations between 0.01 and 1.20 mg mL^{-1}. The relevant data for obtaining the calibration curves is shown in Tale 3.1 Quantification of individual compounds (Table 3.3) was obtained using the calibration data of the most similar standard. Three aliquots of the extract were injected in triplicate, and compound concentrations were the average value calculated in each case.

Table 3.1 – Calibration data used for the HPLC-UV quantification of phenolic components of *E. globulus* bark extracts

Compound	λ (nm)	Conc. Range (mg mL^{-1})	Calibration curve[a]	R^2	LOD[b]	LOQ[c]
Gallic acid	280	0.01 – 0.60	y = 535985x+1092	0.999	0.021	0.069
Protocatechuic acid	280	0.05 – 1.20	y = 527991x-5289	1.000	0.029	0.096
Catechin	280	0.01 – 1.20	y = 100865x+2057	1.000	0.028	0.092
Chlorogenic acid	280	0.01 – 0.80	y = 229162x-1450	0.999	0.029	0.097
Caffeic acid	280	0.01 – 0.33	y = 992484x-1804	0.997	0.023	0.075
Ellagic acid	340	0.01 – 0.44	y = 300168x+3045	0.998	0.023	0.076
Quercetin	340	0.01 – 0.33	y = 619494x+2454	0.999	0.014	0.046
Naringenin	280	0.01 – 0.28	y = 722267x+1939	0.998	0.016	0.052

[a] y = peak area, x = concentration in mg mL^{-1}; [b] LOD: limit of detection, expressed in mg mL^{-1}; [c] LOQ: limit of quantification, expressed in mg mL^{-1}

3.3 Results and discussion

3.3.1 Extraction yields and total phenolic content

The extraction yield of *E. globulus* bark obtained with MeOH/H_2O (9.28%) is lower than the sum of the extraction yields for MeOH (8.24%) and water (1.93%) (Table 3.2). The MeOH and MeOH/H_2O extraction yield values reported here are considerably higher than those recently published for *E. globulus* bark extracts [16]; however, the extraction conditions applied were different, including the temperature, time of extraction, and solid/liquid ratio. The total phenolic contents of the three extracts of *E. globulus* bark (Table 3.2), determined by Folin-Ciocalteu method, accounted for 115.3±0.50, 409.7±2.76, and 413.8±5.27 mg GAE g^{-1} in water, MeOH, and MeOH/H_2O extracts, respectively, demonstrating that MeOH and MeOH/H_2O extracts have similar TPCs and

that, jointly, in the sequential extraction with MeOH followed by water, this last solvent adds 20% more TPC in comparison to the single step extraction with MeOH/H_2O. Finally, the reported phenolic contents are in the range of those previously reported for *E. globulus* bark extracts [16].

Table 3.2 – Extraction yield and total phenolic content of *E. globulus* bark extracts

Extracts	Extraction yield (%)	Total phenolic content (mg GAE[a] g^{-1} of extract)
MeOH	8.24	409.7 ± 2.76
H_2O	1.93	115.3 ± 0.50
MeOH/H_2O	9.28	413.8 ± 5.27

[a] GAE: gallic acid equivalents

3.3.2 Identification of phenolic compounds

The identification of the components of the MeOH, H_2O, and MeOH/H_2O extracts was carried out by HPLC–UV, HPLC-MS/MS, and, in some cases, MS^n using distinct equipment, as described above. The UV chromatogram of MeOH/H_2O extract recorded at 340 nm is shown in Figure 3.1

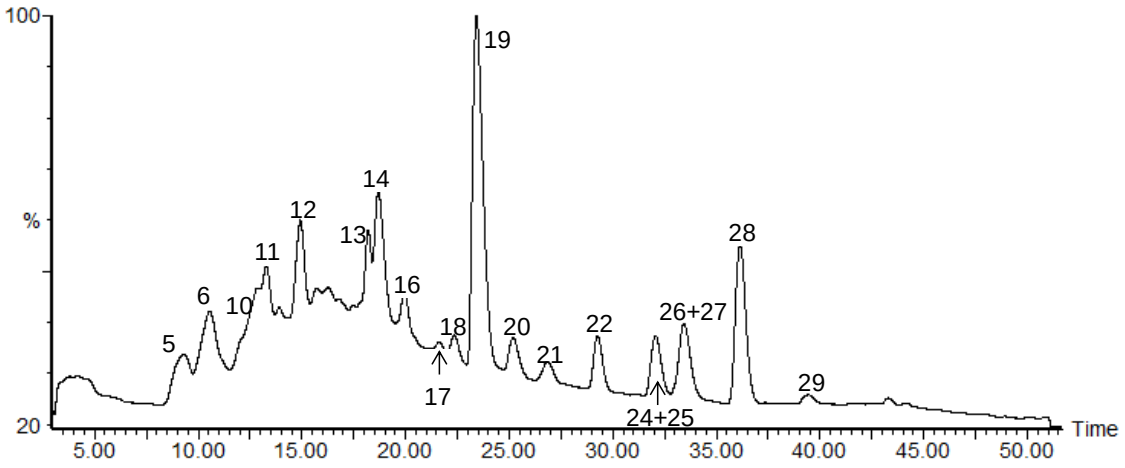


Figure 3.1 – HPLC-UV chromatogram of MeOH/H_2O extract of *E. globulus* bark at 340 nm

Table 3.3 summarizes the phenolic compounds characterised in each extract, their retention time, the molecular ion [M–H]-, and the main product ions obtained by HPLC–MS/MS and in some cases by MS^n. Compounds were identified by comparing their fragmentation profiles with reference compounds run under the same experimental conditions, or, when standards were not available, their identifications were corroborated with the literature as indicated in Table 3.3 and discussed bellow.

Table 3.3 – Phenolic compounds identified in *E. globulus* bark extracts and corresponding MS/MS and MS^n fragmentation profiles

Peak no.	Rt (min)	Compound	$[M–H]^-$ (*m/z*)	QqQ–MS/MS product ions (*m/z*)	IT–MS^n product ions (*m/z*)	Identified	Presence in *Eucalyptus* species[d]
1	3.2	Quinic acid[a]	191	173, 111, 87, 85	MS^2: 173, 149, 127, 111, 93, 85	[17]	RFE
2	3.8	Gallic acid	169	125	-	Co[b]	Egl b, w, l, f, Eca Eru l, Ere b, w [18]
3	5.1	dihydroxyphenylacetic acid[a]	167	123	-	[19]	RFE
4	5.7	Protocatechuic acid	153	109	-	Co	Egl b [8] Egl Eca Eru b [20], l [21]
5	10.1	bis-HHDP–glucose[a]	783		MS^2: **481**[c], 301; MS^3: 301, 275	[18]	Egl f, Eni w, Eco Evi l [18]
6	11.2	Methyl gallate	183	168, 124	-	[22]	Egl b [23]
7	12.4	Catechin	289	245, 205, 203, 125, 109	-	Co	Egl l [11], b [10, 23], w [6], Ere b, w [6]
8	13.2	Chlorogenic acid	353	191	-	Co	Egl b, w [6] Ecy l [24]
9	13.9	Galloyl-bis-HHDP–glucose[a]	935	935, 633, 301, 300, 275	MS^2: **633**; MS^3: 481, 299, 275	[18, 25]	Egl f, Eni w Eal f [18]
10	14.4	Galloyl-HHDP–glucose[a]	633	301	MS^2: 615, 481, 463, 421, **301**, 275; MS^3: 257, 229	[18]	Egl f, Eni w Eco l [18]
11	14.9	Caffeic acid[a]	179	135	-	Co	Ehy l [26]
12	15.6	Digalloylglucose	483	313, 169	MS^2: 331, 327, **313**, 169; MS^3: 271, 211, 193, 169, 125	[18]	Egl b [10], Egl f, Eni w Eco l [18]
13	19.3	Isorhamnetin-hexoside[a]	477	315	MS^2: **315**; MS^3: **300**; MS^4: 271, 272, 244	[27]	RFE
14	19.8	Ellagic acid	301	229, 185, 173, 157, 146	-	Co	Esp b, f, l, w [18]
15	20.7	Taxifolin	303	285, 177, 151, 125	-	[28]	Egl b [8, 20, 23]
16	21.7	Quercetin-hexoside[a]	463	301, 300	MS^2: **301**; 300; MS^3: 179, 151	[29]	Egl f, l, Eca Eru l Egu h [18]
17	22.9	Methyl-ellagic acid-pentoside[a]	447	315	MS^2: **315**; MS^3: **300**	[18]	Egl f [18]

Peak no.	Rt (min)	Compound	$[M–H]^-$ (*m/z*)	QqQ–MS/MS product ions (*m/z*)	$IT–MS^n$ product ions (*m/z*)	Identified	Presence in *Eucalyptus* species[d]
18	23.8	Myricetin-rhamnoside[a]	463	317	-	[30]	RFE
19	24.9	Isorhamnetin-rhamnoside[a]	461	315	MS^2: **315**; MS^3: **300**; MS^4: 272, 244	[31]	RFE
20	26.6	Aromadendrin-rhamnoside	433	287, 269, 259, 180, 179, 151	MS^2: **287**; 269; MS^3: **259**; MS^4: 241, 215, 125	[28]	Egl b [23]
21	28.4	Mearnsetin[a]	331	316	MS^2: **316**; MS^3: **287**, 271; MS^4: 259	[32]	RFE
22	29.4	Phloridzin[a]	435	273	MS^2: **273**; MS^3: **167**; MS^4: 123	[33]	RFE
23	30.9	Mearnsetin-hexoside[a]	493	331	MS^2: **331**; MS^3: **316**; MS^4: **287**, 271, 244; MS^5: 259	[32]	RFE
24	33.1	Eriodictyol	287	151, 135, 107	-	[28]	Egl b [8, 23]
25	33.8	B-type proanthocyanidin dimer[a]	577		MS^2: 451, 425, 407, **289**, 287; MS^3: 245, 205	[30, 34]	RFE
26	34.8	Luteolin[a]	285	175, 151, 133	-	Co	Egl Eca l [21], Eca b [20]
27	35.2	Quercetin	301	179, 165, 151, 121, 107	-	Co	Egl b [8, 23]
28	37.7	Isorhamnetin	315	300, 272, 271	-	[27]	Egl b [8, 10]
29	40.5	Naringenin	271	177, 151, 119, 107	-	Co	Egl b [8, 20], Eca b [20], Eru l [21]

[a]compounds identified for the first time in *E. globulus* bark; [b]Co = co-injection of the authentic standard; [c]*m/z* in bold was subjected to MS^n analysis; [d] n: *Egl- E. globulus, Eca- E. camaldulensis, Eru- E. rudis, Ere- E. regnans, Eni-E. nitens, Eco- E. consideniana, Evi- E. viminalis, Ecy- E. cypellocarpa, Egu- E. gunnii, Eal-E. alba, Ehy- Eucalyptus* hybrids, Esp- *Eucalyptus* species; b-bark, l-leaves, f-fruits, w-wood, h-hook; RFE- Reported for the first time in *Eucalyptus* species

3.3.2.1 Phenolic acids and esters

Compound **1** was assigned to quinic acid, due to its $[M-H]^-$ ion at *m/z* 191 yielding characteristic product ions at *m/z* 173, 111, 87 and 85, upon dissociation [17]. Compounds **2**, **4** and **9** were identified as gallic, protocatechuic and caffeic acids (Figure 3.2), respectively, by comparing their retention times and fragmentation pathways observed in the MS/MS spectra (QqQ) with those of the corresponding reference compounds. These phenolic compounds showed the typical loss of the carboxylic group (-44 Da, $-CO_2$). Compound **3** was identified as dihydroxyphenylacetic acid (Figure 3.2), due to its $[M-H]^-$ ion at *m/z* 167, that cleaves, under MS/MS conditions, with formation of the ion at *m/z* 123, indicating the loss of a carboxylic group (-44 Da, $-CO_2$) [19].

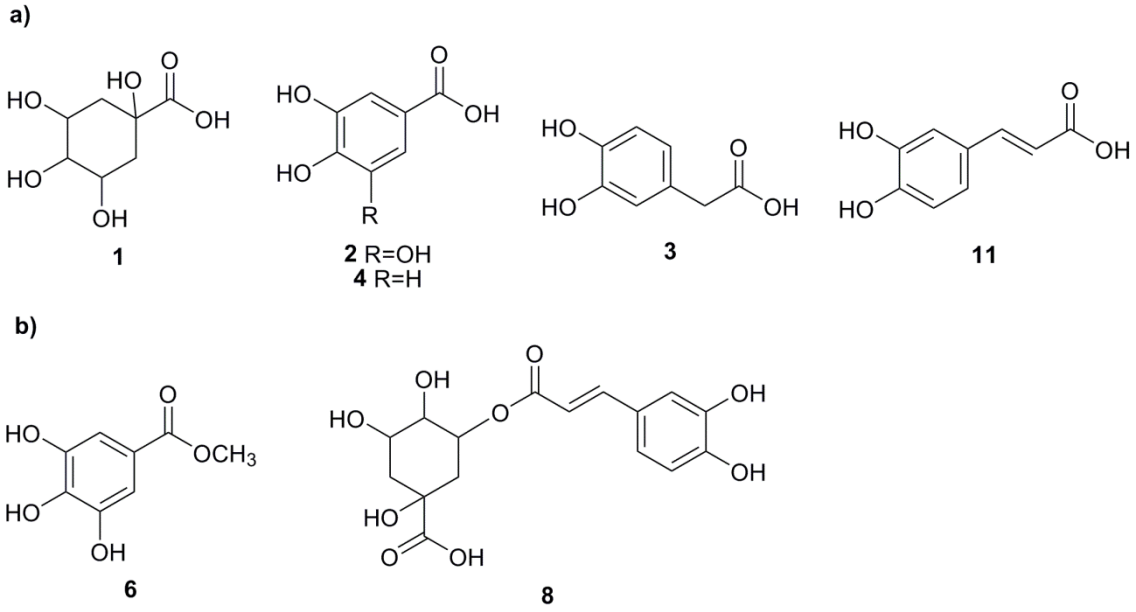


Figure 3.2 – Structures of a) phenolic acids and b) phenolic esters identified in *E. globulus* bark

Peak **6** matches with methyl gallate (Figure 3.2), with $[M-H]^-$ ion at *m/z* 183, which, upon dissociation shows characteristic product ions at *m/z* 168 and 124, due to the consecutive loss of the methyl group (-15 Da, $-CH_3$) and of the carboxylic acid group (-44 Da, $-CO_2$), respectively [22]. Figure 3.3 shows the MS/MS spectrum (QqQ) and the fragmentation pathway of this phenolic ester.

The fragmentation pathways observed and the retention time of compound **8** matched to those of an authentic standard of chlorogenic acid, for which the main product ion is observed at *m/z* 191, assigned to the quinic acid moiety.

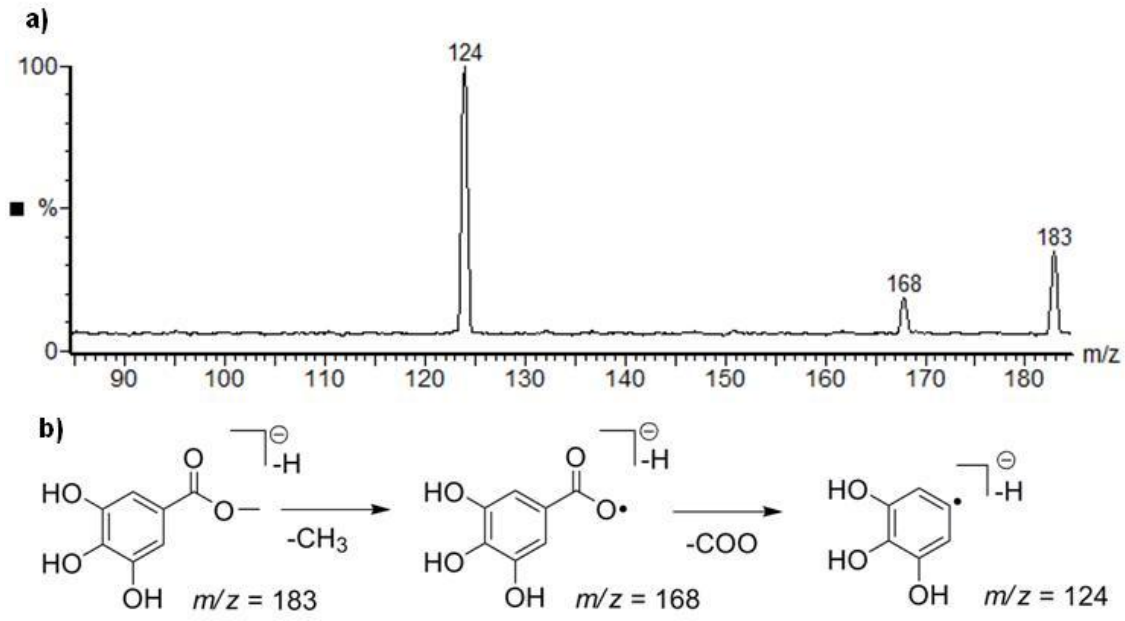


Figure 3.3 – a) MS/MS spectrum and b) fragmentation pathways of methyl gallate **6**

3.3.2.2 Flavonoids

Flavonoid fragmentation pathways are recognized by the typical retro-Diels-Alder fissions [35], the main product ions of which observed in this study are shown in Figure 3.4, using the nomenclature adapted from that proposed by Ma *et al*. [36].

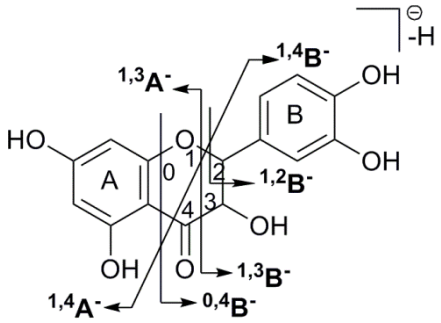


Figure 3.4 – Fragmentation nomenclature for $[M–H]^-$ flavonoids (adapted from [35, 36]). The superscripts on the left of the A or B ring indicate the broken C-ring bonds.

Flavanols: Compound **7** corresponds to catechin (Figure 3.5), as confirmed by co-injection with a standard. Its $[M-H]^-$ ion, at *m/z* 289, and the respective fragmentation pathway of this flavan-3-ol, with formation of the ions at *m/z* 245 $[M-H-CO_2]^-$ and at *m/z* 205, 125 $[^{1,4}A]$- and 109 (B ring), correspond to typical cleavage of flavonoid rings [35]. Compound **25** was identified as a B-type proanthocyanidin dimer (Figure 3.5) with $[M-H]^-$ ion at *m/z* 577. The corresponding MS^n spectra show the product ions at *m/z* 425, with a loss of 152 mass units, characteristic of the retro-Diels-Alder fission [34], at *m/z*

407 [M-H-$C_8H_8O_3$-H_2O]$^-$, and also the two product ions at *m/z* 289 and 287, which correspond to the two flavanol monomeric units [28, 30]. Furthermore, the MS^3 of the ion at *m/z* 289 generates the characteristic product ions of catechin.

Flavonols: Peak **21** was assigned to free mearnsetin (Figure 3.5), based on its [M-H]$^-$ at *m/z* 331 and on its fragmentation patterns, showing a loss of a methyl group (product ions at *m/z* 316) and the typical cleavage of the flavonoid moiety during fragmentation (product ions at *m/z* 287 [M-H-CH_3-HCO]$^-$ and 259 [M-H-CH_3-HCO-CO]$^-$) [32]. Peak **27** was identified as quercetin (Figure 3.5), based on the matching of the retention time, as well as the fragmentation pattern, with those of an authentic standard. Peak **28** was identified as isorhamnetin (Figure 3.5), based on the dissociation of its [M-H]$^-$ ion at *m/z* 315, which generates the product ions at *m/z* 300 [M-H-CH_3]$^{-\cdot}$and 272 [M-H-CH_3-CO]$^-$, characteristic of this compound [27].

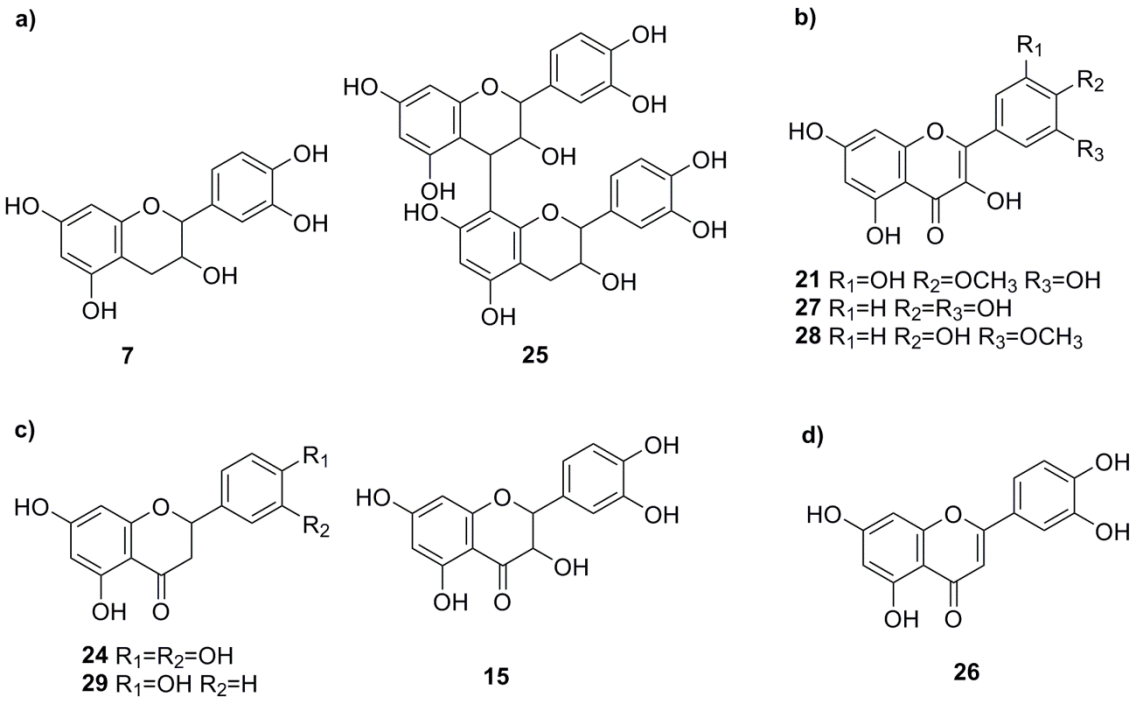


Figure 3.5 – Structures of a) flavanols, b) flavonols, c) flavanones, d) flavones identified in *E. globulus* bark

Flavones: Compound **26** was identified as luteolin (Figure 3.5), based on the [M-H]$^-$ at *m/z* 285, on the product ions observed in the MS/MS spectrum at *m/z* 175 [M-H-C_3O_2-C_2H_2O]$^-$, 151 [^{1,3}A]$^-$ and 133 [^{1,3}B]$^-$ and on the retention time, that matched to authentic standard.

Flavanones: Peak **15** was identified as taxifolin (Figure 3.5), due to its [M-H]$^-$ ion at *m/z* 303 and the subsequent fragmentation, that yield ions at *m/z* 285 [M-H-H_2O]$^-$, 177 [M-

H-ring B]$^-$, 151 [^{1,3}A]$^-$ and 125 [^{1,4}A]$^-$, corresponding to the typical fragmentation pathways of flavonoids [35]. Peak **24** was assigned as eriodictyol (Figure 3.5), based on the [M-H]$^-$ ion at *m/z* 287 and on its dissociation, that leads to the ion at *m/z* 151 [^{1,3}A]$^-$, typical of flavonoid-type structures, resulting from a retro Diels-Alder cleavage of the heterocyclic ring C [28, 35]. Peak **29** was identified as naringenin after comparing its [M-H]$^-$ ion at *m/z* 271, the corresponding fragmentation to the ions at *m/z* 177 [M-H-ring B]$^-$, 151 [^{1,3}A]$^-$, 119 [^{1,3}B]$^-$ and 107 [^{1,3}A-CO_2]$^-$, and its retention time with those of an authentic standard.

3.3.2.3 Flavonoid glycosides

Compound **13** was identified as isorhamnetin-hexoside (Figure 3.6), based on its [M-H]$^-$ at *m/z* 477 and on the corresponding loss of 162 mass units, which corresponds to the loss of a glucose or galactose unit, and the ion at *m/z* 315, corresponding to isorhamnetin aglycone [27]. The MS^n spectra and fragmentation pathway of isorhamnetin-hexoside is shown in Figure 3.7.

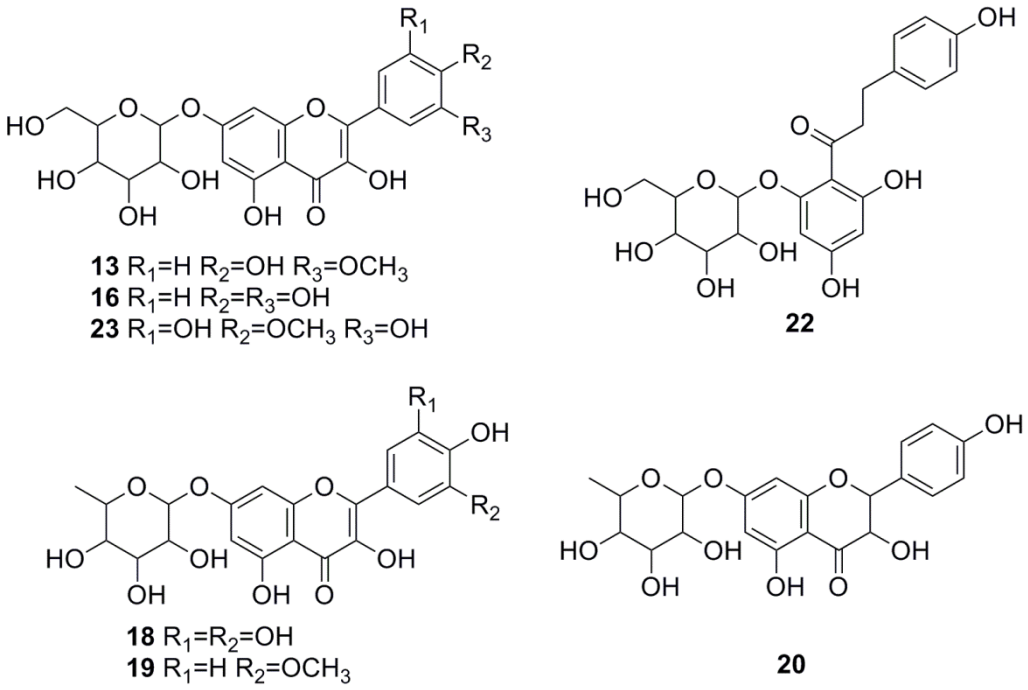


Figure 3.6 – Structures of flavonoid glycosides identified in *E. globulus* bark

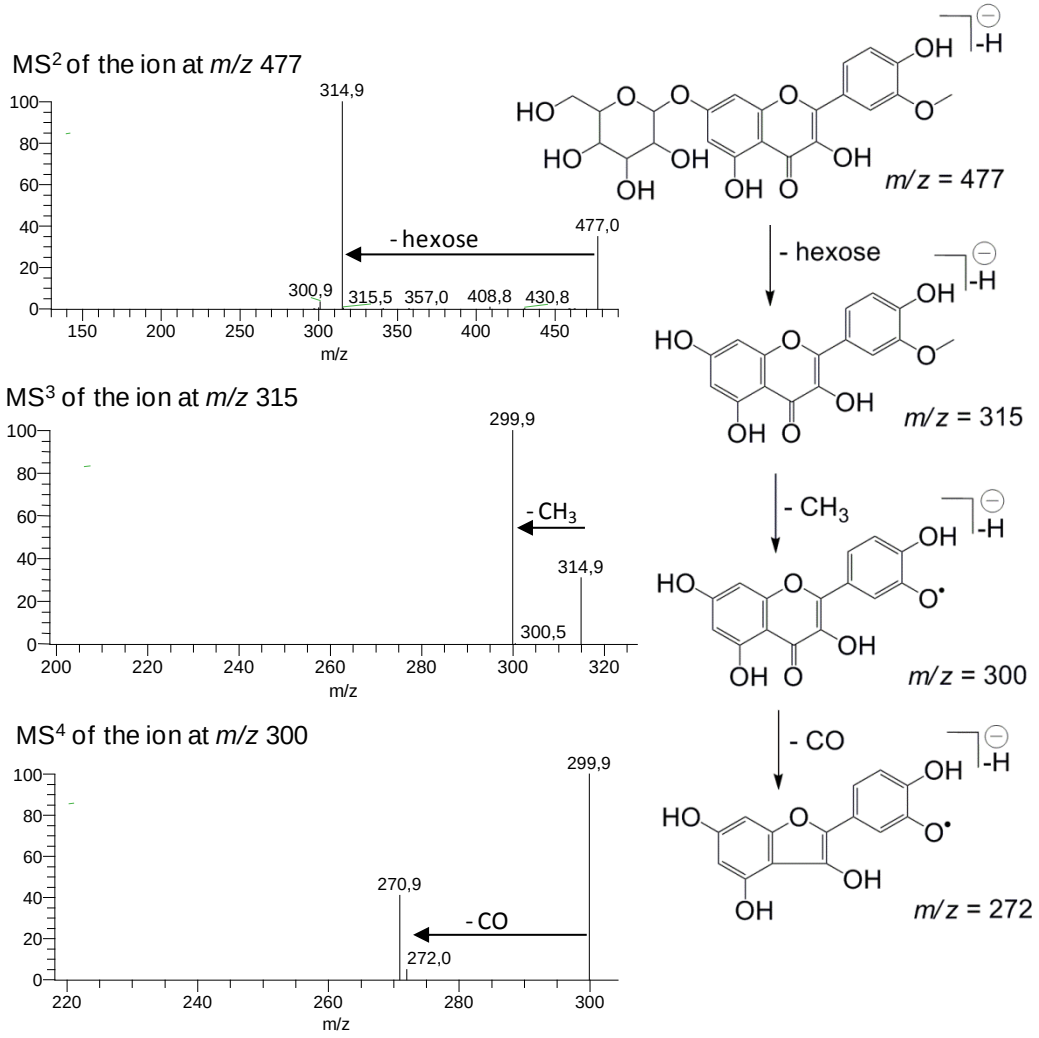


Figure 3.7 – MS^n spectra (left) and fragmentation pathway (right) of isorhamnetin-hexoside **13**

Peak **16** was identified as quercetin-hexoside (Figure 3.6), since the MS^2 of the $[M-H]^-$ at *m/z* 463 generates the ion at *m/z* 301, evidently produced by the loss of an hexose moiety $[M-H-162]^-$, which could correspond to a galactoside or glucoside unit [29]. On the other hand, the dissociation of the aglycone, observed in the MS^3 spectrum, yielded ions at *m/z* 179 and 151, corresponding to the fragmentation pathways of a quercetin standard (as described above). The MS^n spectra and fragmentation pathways of isorhamnetin-hexoside is shown in Figure 3.5. Peak **18** is proposed to correspond to myricetin-rhamnoside (Figure 3.6), since the fragmentation pathway observed for the $[M-H]^-$ ion at *m/z* 463 $[M-H]^-$ shows the loss of a rhamnose unit and the ion generated correspond to the myricetin aglycone [30]. Peak **19** is assigned to isorhamnetin-rhamnoside (Figure 3.6), with its $[M-H]^-$ at *m/z* 461 showing the loss of 146 Da, which indicate the presence of a rhamnose sugar unit. The MS^3 (315→300) and MS^4 (300→272, 244) fragmentation pathways obtained by IT-MS also correspond to the isorhamnetin aglycone (as described above). Likewise, peak **20** was assigned to

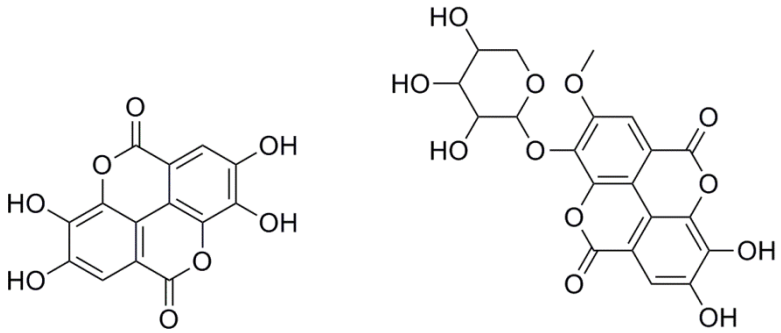
aromadendrin-rhamnoside, with its $[M-H]^-$ at *m/z* 433 generating, in the MS^2 spectrum, the ion at *m/z* 287, due to the loss of a rhamnose unit $[M-H-146]^-$. Furthermore, the MS^n spectra of the ion at *m/z* 287, obtained in the ion trap, show the typical fragmentation of aromadendrin aglycone, generating the product ions at *m/z* 259 $[M-H-CO]^-$ and 125 $[^{1,4}A]^-$ [28].

Compound **22** was identified as phloridzin (Figure 3.6), the glucosidic form of phloretin, with its $[M-H]^-$ ion at *m/z* 435 yielding the product ion at *m/z* 273, corresponding to the loss of the glucose unit $[M-H-162]^-$. In addition, the MS^3 of the aglycone at *m/z* 273 shows the typical loss of the B ring unit, generating the product ion at *m/z* 167 [33]. Peak **23** was assigned to mearnsetin-hexoside (Figure 3.6), with its molecular ion at *m/z* 493 dissociating to a product ion at *m/z* 331, by the loss of a hexose unit. The MS^n of the aglycone obtained by IT–MS, until MS^5, shows the characteristic dissociation of mearnsetin (493→331→316→287, 271; 287→259), as described above [32].

Although some authors reported the possibility of differentiating flavonoid glycoside positional isomers on the basis of mass spectrometry data [12, 29], such differentiation was not unambiguously possible in the present study. However, the 7-*O*-glycosidic unit linkage, one of the most typical [12], was assumed for the structures presented in Figure 3.4.

3.3.2.4 Ellagic acid and derivatives

Compound **14** was identified as ellagic acid (EA) (Figure 3.8), after comparison of its retention time and fragmentation pathways with those of an authentic standard.



14 **17**

Figure 3.8 – Structures of ellagic acid **14** and methyl-ellagic acid-pentoside **17**

Compound **17** shows an $[M–H]^-$ ion at *m/z* 447, which, on the basis of the molecular weight, could be either a methyl-EA-pentoside, an EA-rhamnoside, or an isorhamnetin-pentoside. However, the presence in the MS^2 and MS^3 spectra of the ions at *m/z* 315

(–132 Da, –pentose) [18] and at *m/z* 300 (–15 Da, $–CH_3$), respectively, allow the identification of this compound as methyl-EA-pentoside (Figure 3.8). Thus, the identification is also confirmed by the absence of the product ions at *m/z* 271 (which excludes the isorhamnetin algycone), and at *m/z* 229 and 185 (which exclude ellagic acid).

3.3.2.5 Galloylglucose derivatives and ellagitannins

Compound **12** was assigned to digalloylglucose isomer (Figure 3.9), with its $[M–H]^-$ at *m/z* 483 showing product ions at *m/z* 313 (loss of a galloyl moiety) and at *m/z* 169 (gallic acid deprotonated ion). In this case, as well as for compound **10**, one of the galloyl units should be linked to C-1, as only a single HPLC peak is observed [37].

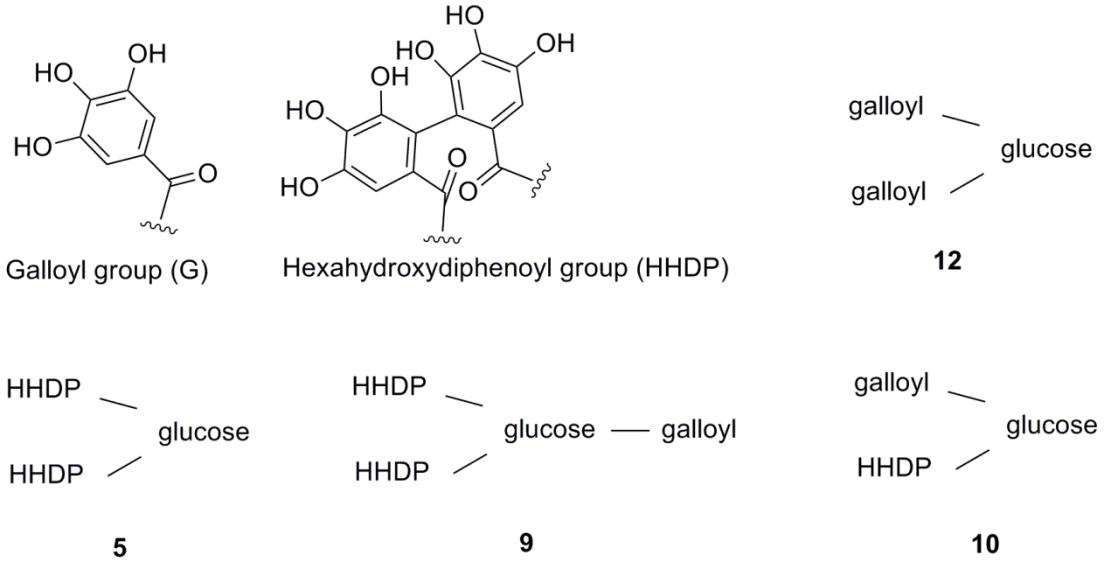


Figure 3.9 – Structures of galloylglucose derivatives and ellagitannins identified in *E. globulus* bark

Compound **5** showed the characteristic fragmentation pathway of an ellagitannin with a $[M–H]^-$ at *m/z* 783 and the MS^2 spectrum showing ions at *m/z* 481 (loss of HHDP) and at *m/z* 301 (loss of HHDP-glucose). This allowed to identify the compound as bis-HHDP-glucose (Figure 3.9) [18]. Identification was corroborated by the MS^3 spectrum (481→301), confirming the loss of a glucose unit from this precursor ion. Compound **9** was identified also as an ellagitannin, a galloyl-bis-HHDP-glucose (Figure 3.9), because its $[M-H]^-$ ion at *m/z* 935 is typical for ellagitannins with this structure. Furthermore, the MS/MS spectrum shows the ions at *m/z* 633 (–302 Da, –HHDP) and at *m/z* 301 $[HHDP–H]^-$, due to the loss of the HHDP and galloyl and glucose units. This identification was corroborated by the MS^3 (633→481), obtained in the ion trap, which shows the loss of a galloyl group [18]. Compound **10** was presumed to be an isomer of

galloyl-HHDP-glucose (Figure 3.9). The fragmentation of the molecular ion $[M–H]^-$ at *m/z* 633 (Figure 3.10) yields the product ions at *m/z* 481, due to the loss of the galloyl group, and at *m/z* 301, corresponding to the HHDP unit after lactonisation to ellagic acid [18].

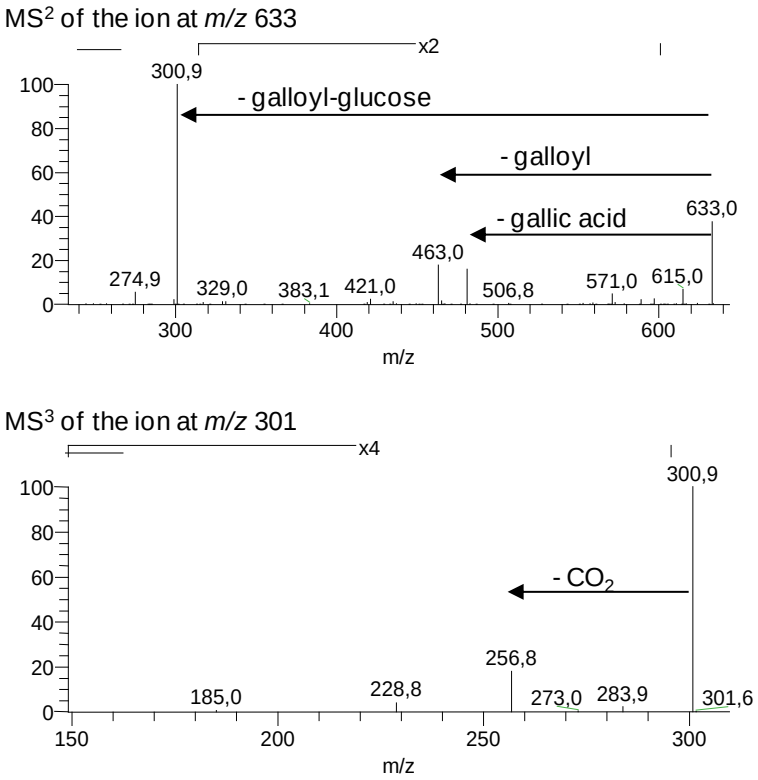


Figure 3.10 – MS^n spectra of galloyl-HHDP-glucose **11**: a) MS^2 of the ion at *m/z* 633; b) MS^3 of the ion at *m/z* 301

The fragmentation pathway of a galloyl-HHDP-glucose isomer is illustrated in Figure 3.11.

To the best of our knowledge, a total of 16 compounds are reported here for the first time as *E. globulus* bark components, namely, quinic **1**, dihydroxyphenylacetic **3** and caffeic **11** acids, bis-HHDP-glucose **5**, galloyl-bis-HHDP-glucose **9**, galloyl-HHDP-glucose **10**, isorhamnetin-hexoside **13**, quercetin-hexoside **16**, methyl-EA-pentoside **17**, myricetin-rhamnoside **18**, isorhamnetin-rhamnoside **19** mearnsetin **21**, phloridzin **22**, mearnsetin-hexoside **23**, luteolin **2**6, and a proanthocyanidin B-type dimer **25**. Although some of these compounds, namely, bis-HHDP-glucose **5**, galloyl-bis-HHDP-glucose **9**, galloyl-HHDP-glucose **10**, quercetin-hexoside **16**, and methyl-EA-pentoside **17**, have already been reported as constituents of other morphological parts of *E. globulus*, such as the leaves and fruits [18], no reference has been made to the bark. Furthermore, proanthocyanidins were already referenced as constituents of *E. globulus*

bark [9], however, B-type dimers have never been reported. In addition, Kim *et al.* [38] and Yazaki *et al.* [6] identified six ellagic acid derivatives in *E. globulus* bark, but none of them as a methyl-EA-pentoside.

Among the phenolic compounds identified for the first time in *E. globulus* bark, eight are reported for the first time as components of the *Eucalyptus* genus (Table 3.3), namely, quinic acid **1**, isorhamnetin-hexoside **13**, myricetin-rhamnoside **18**, isorhamnetinrhamnoside **19**, mearnsetin **21**, phloridzin **22**, mearnsetin-hexoside **23**, and a B-type proanthocyanidin dimer **25**. Finally, to our knowledge, the detailed phenolic composition of the MeOH/H_2O (50:50) extract of *E. globulus* bark is reported here for the first time, although its total phenolic content and antioxidant activity have been previously reported [16].

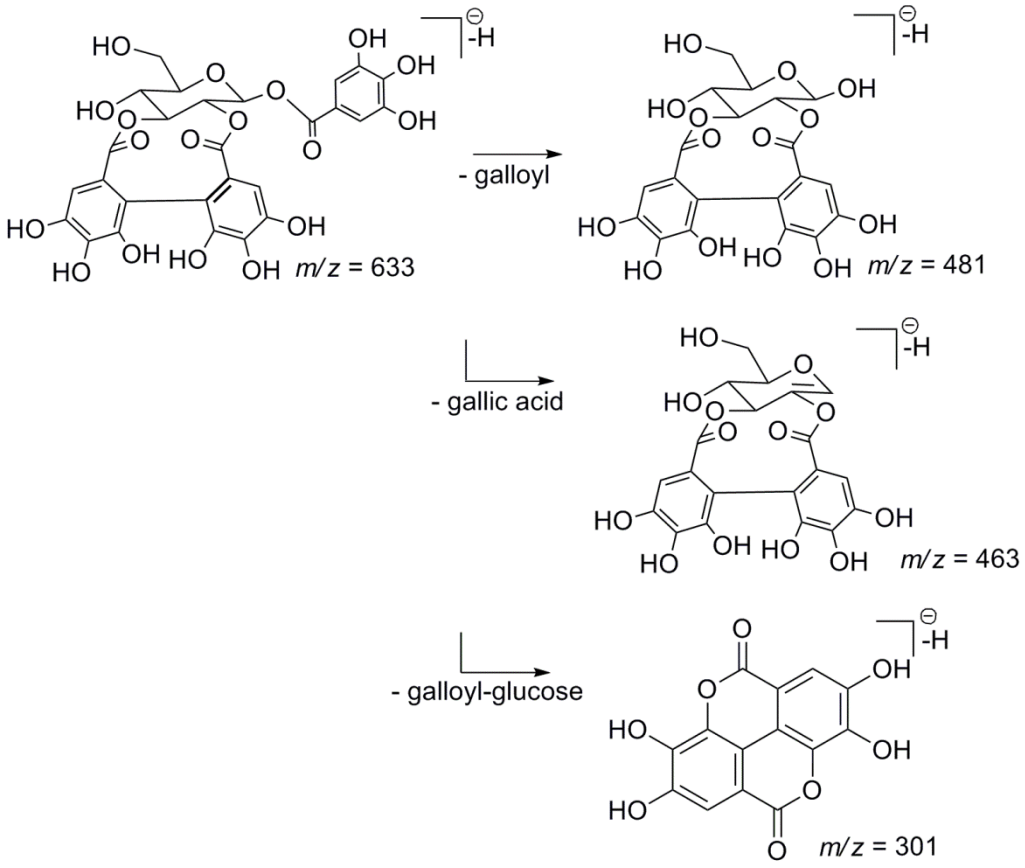


Figure 3.11 – Fragmentation pathway of an isomer of galloyl-HHDP-glucose **10** [18]

3.3.3 HPLC quantification of phenolic compounds

The phenolic content of each extract quantified by HPLC, expressed in mg g^{-1} of extract and in mg kg^{-1} of bark, is shown in Table 3.4. In relation to the extracts composition, the single MeOH/H_2O extraction step shows abundances of the identified compounds in the extracts globally higher than those obtained with MeOH, but lower

than the total obtained in the sequential extraction with MeOH followed by water. However, the total amount of identified compounds per kilogram of bark is clearly higher in the case of the MeOH/H_2O extract (~10.9 g kg^{-1}), when compared to the sum of the other two (~8.4 g kg^{-1}), demonstrating, clearly, the advantage of this single step extraction.

This study showed that digalloylglucose **12** is the main compound in the MeOH and MeOH/H_2O extracts, with values of 17.95 and 17.77 mg g^{-1} of extract, respectively, followed by isorhamnetin-rhamnoside **19** (9.79 mg g^{-1} of extract) and galloyl-HHDP-glucose **10** (9.27 mg g^{-1} of extract) in the MeOH extract, and by catechin **7** (14.23 mg g^{-1} of extract) and chlorogenic acid **8** (13.36 mg g^{-1} of extract) in the MeOH/H_2O extract. It should be highlighted that the same compounds were detected in both MeOH and MeOH/H_2O extracts.

The water extract was found to have considerably lower amounts of phenolic compounds, with catechin **7** (15.94 mg g^{-1} of extract), galloyl-hexahydroxydiphenoyl-glucose **10** (9.04 mg g^{-1} of extract), and digalloylglucose **12** (6.35 mg g^{-1} of extract) as the major components.

These results allow verification that MeOH/H_2O extraction shows to be the most promise to extract phenolic compounds from *E. globulus* bark, as, in general, it allows in a single step higher extraction yields of identified compounds per mass of bark. In contrast with the results obtained, ellagic acid has been previously reported as the major component on the ethyl ether extracts after MeOH/H_2O (80:20) extraction of *E. globulus* bark [8, 20], however, in lower quantities than detected in this study for MeOH and MeOH/H_2O (~5 mg g^{-1} of extract). In fact, the abundance of this compound is considerably lower than those mentioned above for the more abundant compounds detected. These quantitative differences in chemical composition could be derived from the well-known variability of *E. globulus* extractives composition [9, 39] with geographic origin, age of the tree, and part of the tree from which the bark was collected, among others, and also the differences in extraction procedures and analytical methodology.

Table 3.4 – Abundance of phenolic components identified in *E. globulus* bark extracts

Peak no.	Compound	λ (nm)	Phenolic content (mg g^{-1} of extract)			Phenolic content (mg kg^{-1} of bark)		
			MeOH	H_2O	MeOH/H_2O	MeOH	H_2O	MeOH/H_2O
1	Quinic acid[a]	280	1.46	2.47	1.50	120.34	47.64	139.51
2	Gallic acid[a]	280	3.41	3.52	8.83	280.84	67.86	819.48
3	dihydroxyphenylacetic acid[a]	280	-	0.38	-	-	7.29	-
4	Protocatechuic acid[b]	280	1.62	2.80	2.09	133.10	53.96	194.14
5	bis-HHDP–glucose[a]	280	0.68	0.83	1.02	56.35	16.06	94.30
6	Methyl gallate[a]	280	0.68	2.43	1.50	56.18	46.95	139.25
7	Catechin[c]	280	6.57	15.94	14.23	541.56	307.75	1320.59
8	Chlorogenic acid[d]	280	5.98	5.24	13.36	492.92	101.04	1239.46
9	Galloyl-bis-HHDP–glucose[a]	280	7.23	4.99	4.85	595.89	96.34	450.37
10	Galloyl-HHDP–glucose[a]	280	9.27	9.04	6.86	764.15	174.42	637.04
11	Caffeic acid[e]	280	-	5.03	-	-	97.02	-
12	Digalloylglucose[a]	280	17.95	6.35	17.77	1479.42	122.64	1648.91
13	Isorhamnetin-hexoside[f]	340	1.53	traces	1.08	126.38	traces	99.89
14	Ellagic acid[g]	340	4.95	traces	5.08	407.99	traces	471.04
15	Taxifolin[h]	280	1.48	-	7.78	121.66	-	721.58
16	Quercetin-hexoside[f]	340	0.15	-	0.63	12.16	-	58.77
17	Methyl-ellagic acid-pentoside[g]	340	traces	-	traces	traces	-	traces
18	Myricetin-rhamnoside[f]	340	0.20	-	0.14	16.41	-	13.31
19	Isorhamnetin-rhamnoside[f]	340	9.79	0.17	10.00	806.57	3.29	927.64
20	Aromadendrin-rhamnoside[h]	280	traces	-	0.79	traces	-	73.52

Peak no.	Compound	λ (nm)	Phenolic content (mg g^{-1} of extract)			Phenolic content (mg kg^{-1} of bark)		
			MeOH	H_2O	MeOH/H_2O	MeOH	H_2O	MeOH/H_2O
21	Mearnsetin[f]	340	0.34	-	0.38	27.76	-	35.30
22	Phloridzin[h]	280	traces	-	0.75	traces	-	69.89
23	Mearnsetin-hexoside[f]	340	1.07	-	1.30	88.10	-	121.12
24	Eriodictyol[h]	280	6.90[i(24+25)]	traces	7.91[i(24+25)]	568.53[i(24+25)]	traces	733.86[i(24+25)]
25	B-type Proanthocyanidin dimer[h]	280		-			-	
26	Luteolin[f]	340	2.31[i(26+27)]	-	3.66[i(26+27)]	190.08[i(26+27)]	-	340.00[i(26+27)]
27	Quercetin[f]	340		-			-	
28	Isorhamnetin[f]	340	3.98	-	4.65	327.80	-	431.63
29	Naringenin[h]	280	0.79	-	0.76	65.17	-	70.92
	Total (mg g^{-1} of extract / mg kg^{-1} of bark)		**88.34**	**59.18**	**116.93**	**7279.34**	**1142.26**	**10851.52**

Results correspond to the average value estimated from the injection of three aliquots analysed in triplicate (standard deviation less of 5%).
Calibrations curved used: [a] Gallic acid, [b] Protocatechuic acid, [c] Catechin, [d] Chlorogenic acid, [e] Caffeic acid, [f] Quercetin, [g] Ellagic acid, [h] Naringenin;
[i] Sum of the phenolic content by partial overlapping

3.4 Conclusions

The phenolic composition of methanol, water, and methanol/water extracts of *Eucalyptus globulus* Labill. bark were analysed and compared. Twenty-nine phenolic compounds were identified, among which, digalloylglucose was the most abundant in methanol and methanol/water extracts, followed by isorhamnetin-rhamnoside in the methanol extract and by catechin in the methanol/water extract. Catechin and galloyl-HHDP-glucose were identified as the predominant components of water extract. Sixteen of the phenolic compounds identified were referenced for the first time as constituents of *E. globulus* bark, namely, quinic, dihydroxyphenylacetic, and caffeic acids, bis-hexahydroxydiphenoyl (HHDP)-glucose, galloyl-bis-HHDP-glucose, galloyl-HHDP-glucose, isorhamentin-hexoside, quercetin-hexoside, methyl-ellagic acid (EA)-pentoside, myricetin-rhamnoside, isorhamnetin-rhamnoside, mearnsetin, phloridzin, mearnsetin-hexoside, luteolin, and a proanthocyanidin B-type dimer. Methanol/water extraction showed to be the most efficient to isolate phenolic compounds identified in *E. globulus* bark.

References

[1] Potts, B. M.; Vaillancourt, R. E.; Jordan, G.; Dutkowski, G.; Silva, J.; McKinnon, G.; Steane, D.; Volker, P.; Lopez, G.; Apiolaza, L.; Li, L. N.; Marques, C.; Borralho, N. In *Eucalyptus in a changing world*, IUFRO Conference, Aveiro, Portugal, 11-15 October, 2004; Borralho, N.; Pereira, J. S.; Marques, C.; Coutinho, J.; Madeira, M.; Tomé, M., Eds. Aveiro, Portugal, 2004.

[2] Quilhó, T.; Pereira, H., Within and between-tree variation of bark content and wood density of *Eucalyptus globulus* in commercial plantations. *IAWA J.* **2001,** *22*, 255-265.

[3] Kamm, B.; Kamm, M.; Gruber, P. R.; Kromus, S., Biorefinery Systems-An overview. In *Biorefineries- Industrial Processes and Products: Status Quo and Future Directions*, B. Kamm, P. G., M. Kamm, Ed. Wiley VCH: Weinheim, Germany, 2006; Vol. 1, pp 3-40.

[4] Freire, C. S. R.; Silvestre, A. J. D.; Neto, C. P.; Cavaleiro, J. A. S., Lipophilic extractives of the inner and outer barks of *Eucalyptus globulus*. *Holzforschung* **2002,** *56*, 372-379.

[5] Domingues, R. M. A.; Sousa, G. D. A.; Freire, C. S. R.; Silvestre, A. J. D.; Pascoal Neto, C., *Eucalyptus globulus* biomass residues from pulping industry as a source of high value triterpenic compounds. *Ind. Crop. Prod.* **2010,** *31*, 65-70.

[6] Yazaki, Y.; Hillis, W. E., Polyphenols of *Eucalyptus globulus*, *E. Regnans* and *E. Deglupta*. *Phytochemistry* **1976,** *15*, 1180-1182.

[7] Fechtal, M.; Riedl, B., Analyse des extraits tannants des écorces des *Eucalyptus* après hydrolyse acide par la chromatographie en phase gazeuse couplée avec la spectrométrie de masse (GC-MS). *Holzforschung* **1991,** *45*, 269-273.

[8] Conde, E.; Cadahía, E.; García-Vallejo, M.; Tomás-Barberán, F., Low molecular weight polyphenols in wood and bark of *Eucalyptus globulus Wood Fiber Sci.* **1995,** *27*, 379-383.

[9] Cadahía, E.; Conde, E.; deSimon, B. F.; GarciaVallejo, M. C., Tannin composition of *Eucalyptus camaldulensis*, *E. globulus* and *E. rudis*. Part II. Bark. *Holzforschung* **1997,** *51*, 125-129.

[10] Vázquez, G.; Santos, J.; Freire, M.; Antorrena, G.; González-Álvarez, J., Extraction of antioxidants from eucalyptus (*Eucalyptus globulus*) bark. *Wood Sci. Technol.* **2012,** *46*, 443-457.

[11] Hou, A. J.; Liu, Y. Z.; Yang, H.; Lin, Z. W.; Sun, H. D., Hydrolyzable tannins and related polyphenols from *Eucalyptus globulus*. *J. Asian Nat. Prod. Res.* **2000,** *2*, 205-212.

[12] Vukics, V.; Guttman, A., Structural Characterization of Flavonoid Glycosides by Multi-Stage Mass Spectrometry. *Mass Spectrom. Rev.* **2008,** *29*, 1-16.

[13] Harborne, J. B., *Phytochemical methods: a guide to modern techniques of plant analysis*. third ed.; Chapman and Hall: London, 1998; p 7-96.

[14] Singleton, V. L.; Rossi, J. A., Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enol. Viticult.* **1965,** *16*, 144-158.

[15] Santos, S. A. O.; Pinto, P.; Silvestre, A. J. D.; Neto, C. P., Chemical composition and antioxidant activity of phenolic extracts of cork from *Quercus suber* L. *Ind. Crop. Prod.* **2010,** *31*, 521-526.

[16] Vázquez, G.; Fontenla, E.; Santos, J.; Freire, M. S.; González-Álvarez, J.; Antorrena, G., Antioxidant activity and phenolic content of chestnut (*Castanea sativa*) shell and eucalyptus (*Eucalyptus globulus*) bark extracts. *Ind. Crop. Prod.* **2008,** *28*, 279-285.

[17] Ng, L. K.; Lafontaine, P.; Vanier, M., Characterization of ciggarette tobacco by direct electrospray ionization-ion trap mass spectrometry (ESI-ITMS) analyis of the aqueous extract - A novel and simple approach. *J. Agric. Food Chem.* **2004,** *52*, 7251-7257.

[18] Boulekbache-Makhlouf, L.; Meudec, E.; Chibane, M.; Mazauric, J. P.; Slimani, S.; Henry, M.; Cheynier, V.; Madani, K., Analysis by High-Performance Liquid Chromatography Diode Array Detection Mass Spectrometry of Phenolic Compounds in Fruit of Eucalyptus globulus Cultivated in Algeria. *J. Agric. Food Chem.* **2010,** *58*, 12615-12624.

[19] Kerwin, J. L., Negative Ion Electrospray Mass Spectrometry of Polyphenols, Catecholamines and their Oxidation Products. *J. Mass Spectrom.* **1996,** *31*, 1429-1439.

[20] Conde, E.; Cadahia, E.; DiezBarra, R.; GarciaVallejo, M. C., Polyphenolic composition of bark extracts from *Eucalyptus camaldulensis*, *E. globulus* and *E. rudis*. *Holz Als Roh- und Werkst.* **1996,** *54*, 175-181.

[21] Conde, E.; Cadahía, E.; García-Vallejo, M. C.; de Simón, B. F.; Adrados, J. R. G., Low molecular weight polyphenols in cork of *Quercus suber*. *J. Agric. Food Chem.* **1997,** *45*, 2695-2700.

[22] Barreto, J. C.; Trevisan, M. T. S.; Hull, W. E.; Erben, G.; de Brito, E. S.; Pfundstein, B.; Wurtele, G.; Spiegelhalder, B.; Owen, R. W., Characterization and quantitation of polyphenolic compounds in bark, kernel, leaves, and peel of mango (*Mangifera indica L.*). *J. Agric. Food Chem.* **2008,** *56*, 5599-5610.

[23] Yun, B.-S.; Lee, I.-K.; Kim, J.-P.; Chung, S.-H.; Shim, G.-S.; Yoo, I.-D., Lipid peroxidation inhibitory activity of some constituents isolated from the stem bark of *Eucalyptus globulus*. *Arch. Pharm. Res.* **2000,** *23*, 147-150.

[24] Ito, H.; Koreishi, M.; Tokuda, H.; Nishino, H.; Yoshida, T., Cypellocarpins A-C, phenol glycosides esterified with oleuropeic acid, from Eucalyptus cypellocarpa. *J. Nat. Prod.* **2000,** *63*, 1253-1257.

[25] Seeram, N. P.; Lee, R.; Scheuller, H. S.; Heber, D., Identification of phenolic compounds in strawberries by liquid chromatography electrospray ionization mass spectroscopy. *Food Chem.* **2006,** *97*, 1-11.

[26] Chapuis-Lard, L.; Contour-Ansel, D.; Bernhard-Reversat, F., High-performance liquid chromatography of water-soluble phenolics in leaf litter of three Eucalyptus hybrids (Congo). *Plant Sci.* **2002,** *163*, 217-222.

[27] Parejo, I.; Jauregui, O.; Sanchez-Rabaneda, F.; Viladomat, F.; Bastida, J.; Codina, C., Separation and characterization of phenolic compounds in fennel (*Foeniculum vulgare*) using liquid chromatography-negative electrospray ionization tandem mass spectrometry. *J. Agric. Food Chem.* **2004,** *52*, 3679-3687.

[28] Sanz, M.; Cadahia, E.; Esteruelas, E.; Munoz, A. M.; Simon, B. F.; Hernandez, T.; Estrella, I., Phenolic Compounds in Cherry (*Prunus avium*) Heartwood with a View to Their Use in Cooperage. *J. Agric. Food Chem.* **2010,** *58*, 4907-4914.

[29] Ablajan, K.; Abliz, Z.; Shang, X. Y.; He, J. M.; Zhang, R. P.; Shi, J. G., Structural characterization of flavonol 3,7-di-O-glycosides and determination of the glycosylation position by using negative ion electrospray ionization tandem mass spectrometry. *J. Mass Spectrom.* **2006,** *41*, 352-360.

[30] Sandhu, A. K.; Gu, L. W., Antioxidant Capacity, Phenolic Content, and Profiling of Phenolic Compounds in the Seeds, Skin, and Pulp of *Vitis rotundifolia* (Muscadine Grapes) As Determined by HPLC-DAD-ESI-MS^n. *J. Agric. Food Chem.* **2010,** *58*, 4681-4692.

[31] Gutzeit, D.; Wray, V.; Winterhalter, P.; Jerz, G., Preparative isolation and purification of flavonoids and protocatechuic acid from sea buckthorn juice concentrate (*Hippophae rhamnoides* L. *ssp rhamnoides*) by high-speed counter-current chromatography. *Chromatographia* **2007,** *65*, 1-7.

[32] Han, J.; Ye, M.; Qiao, X.; Xu, M.; Wang, B. R.; Guo, D. A., Characterization of phenolic compounds in the Chinese herbal drug *Artemisia annua* by liquid chromatography coupled to electrospray ionization mass spectrometry. *J. Pharmaceut. Biomed.* **2008,** *47*, 516-525.

[33] Sanchez-Rabaneda, F.; Jauregui, O.; Lamuela-Raventos, R. M.; Viladomat, F.; Bastida, J.; Codina, C., Qualitative analysis of phenolic compounds in apple pomace using liquid chromatography coupled to mass spectrometry in tandem mode. *Rapid Commun. Mass Spectrom.* **2004,** *18*, 553-563.

[34] Friedrich, W.; Eberhardt, A.; Galensa, R., Investigation of proanthocyanidins by HPLC with electrospray ionization mass spectrometry. *Eur. Food Res.Technol.* **2000,** *211*, 56-64.

[35] Fabre, N.; Rustan, I.; de Hoffmann, E.; Quetin-Leclercq, J., Determination of flavone, flavonol, and flavanone aglycones by negative ion liquid chromatography electrospray ion trap mass spectrometry. *J. Am. Soc. Mass Spectr.* **2001,** *12*, 707-715.

[36] Ma, Y. L.; Li, Q. M.; VandenHeuvel, H.; Claeys, M., Characterization of flavone and flavonol aglycones by collision-induced dissociation tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **1997,** *11*, 1357-1364.

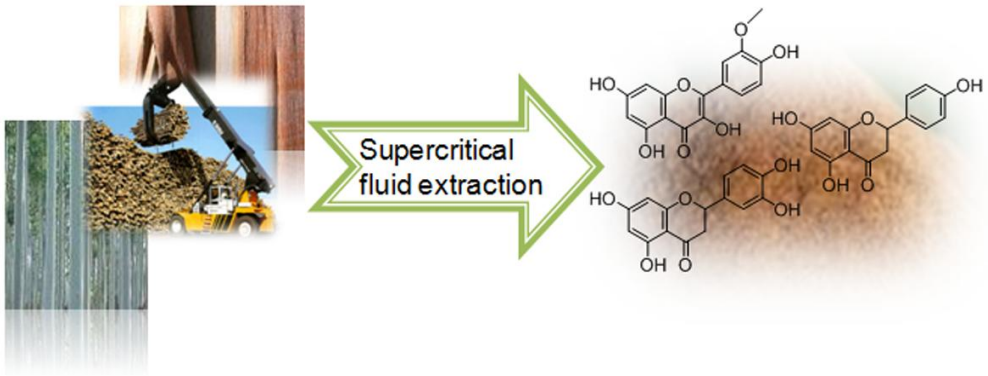
[37] Hatano, T.; Yoshida, T.; Okuda, T., Chromatography of tannins : III. Multiple peaks in high-performance liquid chromatography of some hydrolyzable tannins. *J. Chromatogr. A* **1988,** *435*, 285-295.

[38] Kim, J. P.; Lee, I. K.; Yun, B. S.; Chung, S. H.; Shim, G. S.; Koshino, H.; Yoo, I. D., Ellagic acid rhamnosides from the stem bark of *Eucalyptus globulus*. *Phytochemistry* **2001,** *57*, 587-591.

[39] Cadahía, E.; Conde, E.; García-Vallejo, M. C.; de Simón, B. F., Gel permeation chromatographic study of the molecular weight distribution of tannins in the wood, bark and leaves of *Eucalyptus* spp. *Chromatographia* **1996,** *42*, 95-100.

Part B

Supercritical fluid extraction of phenolic compounds from *Eucalyptus globulus* Labill. bark



Adapted from:

Santos, S.A., Villaverde, J.J., Silva, C.M., Neto, C.P., Silvestre, A.J.D., Supercritical fluid extraction of phenolic compounds from *Eucalyptus globulus* Labill. bark, *Journal of Supercritical Fluids*, **2012**, 71, 71-79

Abstract

The supercritical fluid extraction (SFE) of phenolic compounds from *Eucalyptus globulus* bark was carried out along with detailed analysis of the extracts for the first time. The extracts were accessed in terms of: (i) extraction yield (EY), (ii) total phenolic content by Folin-Ciocalteu method (TPC), (iii) phenolic compounds quantified by high-performance liquid chromatography (PC-HPLC), and (iv) antioxidant activity (AA). Preliminary runs were performed with pure and modified CO_2, using ethanol (EtOH), ethyl acetate, and water. The CO_2/EtOH mixture provided the best extraction yield, high total phenolic content and antioxidant activity. Results also demonstrated high selectivity of the CO_2/EtOH mixture to the extraction of flavanones, particularly eriodictyol and naringenin; and to the *O*-methylated flavonol isorhamnetin, also found in abundance. To analyse the influence of temperature, ethanol content and flow rate on SFE at 300 bar using CO_2/EtOH, a full 2^3 design of experiments was accomplished. The most favourable conditions under the experimental range studied were found as 70 ºC, 20% ethanol, and 10 g CO_2 min^{-1}, for which EY = 0.48%, TPC = 57.22 mg of gallic acid equivalents (GAE) g^{-1} of extract, PC-HPLC = 119.46 mg g^{-1} of extract, and AA = 49.74 mg of ascorbic acid equivalents (AAE) g^{-1} of extract.

3.5 Introduction

Pulp industries generate large amounts of biomass residues, particularly bark, which are simply burned for energy production, which in the case of *E. globulus* represents around 11% of the stem dry weight [1]. The enormous potential for the up-grading of this unexploited renewable resource lead in the last years to several studies about its composition. The lipophilic fraction of *E. globulus* outer bark has shown to be quite rich in high value triterpenic acids, such as ursolic and oleanolic acids [2, 3]. The phenolic fraction of *Eucalyptus* bark has also been attracting interest, mostly due to the wide variety of the well known properties of these compounds, including antioxidant, anti-inflammatory, antithrombotic, among others [4]. Recently a detailed study on the phenolic composition of *E. globulus* bark was done, by carrying out conventional solid-liquid extractions followed by HPLC-MS analyses of the extracts. This approach allowed the identification of 29 phenolic compounds, 16 of which were reported for the first time as *E. globulus* bark constituents (Chapter 3-Part A) [5]. Furthermore, the detailed composition of several other economically important *Eucalyptus* species like *E. grandis*, *E. urograndis* and *E. maidenii* were also published [6].

The extraction of phenolic fractions is normally accomplished with methanol:water mixtures, which enables high gravimetric yields but fairly non selective and complex extracts. Therefore, the utilization of more selective and environmentally friendly separation techniques is welcome in order to increase the value of these extracts. Nowadays, the supercritical fluid extraction (SFE) using carbon dioxide is widely used as a sustainable process in wide variety areas, including the food industry, pharmacy and environmental engineering [7]. However, due to its non-polarity, CO_2 is not efficient for polar (e.g. phenolic compounds) molecules extraction. The introduction of modifiers, such as ethanol, methanol or water, enhances the solvating power of CO_2, which may increase the selectivity and extraction yield of target compounds [8-10].

In this work one focuses the supercritical fluid extraction of phenolic compounds of *E. globulus* bark using carbon dioxide modified with ethanol, ethyl acetate, and water. Results are compared with those obtained by conventional solid-liquid extraction with methanol:water and ethanol:water mixtures. From preliminary results, the most favourable co-solvent was selected and then utilised in the experiments carried out to optimise the SFE of phenolic compounds via statistical design of experiments. The efficiency of all processes was evaluated based on extraction yield (EY), total phenolic

content by Folin-Ciocalteu method (TPC), antioxidant activity (AA), and individual phenolic compounds quantified by HPLC (PC-HPLC).

3.6 Materials and methods

3.6.1 Chemicals

Carbon dioxide was purchased from Praxair (Portugal). Dichloromethane (99% purity), gallic acid (purity higher than 97.5%), quercetin (purity higher than 98%), Folin-Ciocalteu's phenol reagent, 3,5-di-*tert*-4-butylhydroxytoluene (BHT) (purity higher than 99%) and 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) were supplied by Sigma Chemical Co (Madrid, Spain). Protocatechuic acid (purity higher than 97%), and naringenin (98% purity) were obtained from Aldrich Chemical Co (Madrid, Spain). Sodium carbonate (99.9% purity) was supplied by Pronalab (Lisbon, Portugal). Formic acid (purity higher than 98%), methanol (purity higher than 99.8%), and ellagic acid (96% purity) were purchased from Fluka Chemie (Madrid, Spain). Ethanol (99.5% purity) was purchased from Panreac Quimica S.A. (Barcelona, Spain). Ethyl acetate (99.6% purity) was supplied by Acros Organic. HPLC-grade methanol, water, and acetonitrile were supplied from Fisher Scientific Chemicals (Loures, Portugal) and further filtered using a Solvent Filtration Apparatus 58061 from Supelco (Bellefonte, PA, USA).

3.6.2 Raw material

E. globulus bark was taken from 16-year-old *E. globulus* trees randomly harvested from a clone plantation cultivated by RAIZ–Forest and Paper Research Institute in Eixo (40º37'13.56''N, 8º34'08.43''W), region of Aveiro, Portugal. *Eucalyptus* bark was air dried until constant weight and grounded to granulometry lower than 2 mm prior to extraction. The dried bark was submitted to soxhlet extraction with dichloromethane for 6 hours to remove the lipophilic components [2, 3].

3.6.3 Solid-liquid extractions

After the dichloromethane extraction, the solid bark residues were suspended (1:100 g mL^{-1}) in methanol:water ($MeOH/H_2O$) or ethanol:water ($EtOH/H_2O$) 50:50 (v/v) mixtures at room temperature for 24 hours under constant stirring [5]. The suspensions were then filtered, the MeOH or EtOH evaporated under vacuum, and the extracts freeze dried.

3.6.4 Supercritical fluid extraction

3.6.4.1 Supercritical fluid apparatus

Supercritical fluid extractions were performed using the pilot unit Helix SFE System supplied by Applied Separations Inc. (Allentown, PA, USA), which is schematically represented in Figure 3.12. It consists in a 5 x10^{-4} m^3 stainless steel extractor, whose temperature is controlled via a thermocouple, a CO_2 pump, a LabAlliance (Malaysia) series 1500 HPLC pump for co-solvent, and a refrigerated stainless steel collection vessel. For each experiment the extraction column was fed with 40 g of bark, previously extracted with dichloromethane. After a static period of 10 minutes, each extraction was carried out until final consumption of 1.8 kg of CO_2. The extracts were collected in ethanol and evaporated under nitrogen.

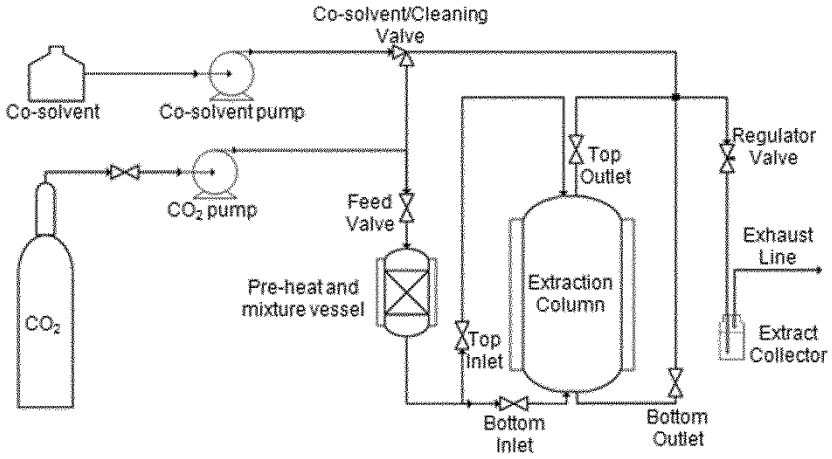


Figure 3.12 – Supercritical fluid extraction unit

3.6.4.2 SFE with pure and modified carbon dioxide

A few preliminary SFE experiments were carried out in order to evaluate the performance of different co-solvents in the removal of our target compounds. The experimental conditions were 300 bar, 70 ºC, and CO_2 flow rate of 8 g min^{-1}, during 225 min. The carbon dioxide was used pure and modified with 15% of ethanol (CO_2/EtOH), 15% of ethyl acetate (CO_2/EtOAc), or 2% of water (CO_2/H_2O). The co-solvents contents were fixed according to literature data for the critical properties of these binary mixtures [11-13].

3.6.4.3 SFE design of experiments

In order to determine the factors that influence the extraction process and the relationships between them, a 2^3 full factorial design was performed with three center points. Temperature (*T*), ethanol content (EtOH), and CO_2 flow rate (*Q*) were the selected variables. The general model for the response Y is the following polynomial:

$$Y=b_0+\sum_{i=1}^{3} b_i X_i + \sum_{i,j=1;i<j}^{3} b_{ij} X_i X_j \quad (3.1)$$

where b_0 is a constant that fixes the response at the central point of the experiment, b_i are the regression coefficients for the linear effect terms, b_{ij} are the coefficients of the interaction effect terms, and X_i represent the normalised independent variables. The coded and uncoded X_i values are shown in Table 3.8. Statistical calculations and analysis were carried out using STATGRAPHICS PLUS 5.1 (1994-2001, Statistical Graphics Corp.).

3.6.5 Analytical methods

3.6.5.1 Extraction yield (EY).

The total mass of each extract was determined gravimetrically in order to calculate the extraction yield.

3.6.5.2 Total phenolic content (TPC)

The experimental procedure to determine the total phenolic content (TPC) can be found in a previous work [5]. The concentration range of extracts involved in this case was 0.08 – 1.40 mg of extract mL^{-1}, whereas for gallic acid standard solutions was 10-85 µg mL^{-1}. Triplicate measurements were carried out.

3.6.5.3 HPLC-UV procedure

The HPLC system consisted of a variable loop Accela autosampler (200 vial capacity set at 15°C), an Accela 600 LC pump and an Accela 80 Hz PDA detector (Thermo Fisher Scientific, San Jose, Ca, USA). Analyses were carried out according to our previously reported procedure [5]. Double online detection was carried out in the diode array detector, at 280 and 340 nm, and UV spectra in a range of 200-600 nm were also recorded. Before the injection, each extract was dissolved in MeOH HPLC grade, to obtain final concentrations between 10 and 20 mg mL^{-1}, and then filtered through a 0.2 µm PTFE syringe filter.

3.6.5.4 ESI–MS^n analysis

The HPLC was coupled to a LCQ Fleet ion trap mass spectrometer (ThermoFinnigan, San Jose, CA, USA), equipped with an electrospray ionization source and operating in negative mode. The nitrogen sheath and auxiliary gas were 40 and 5 (arbitrary units), respectively. The spray voltage was 5 kV and the capillary temperature, 300°C. The capillary and tune lens voltages were set at -28 V and -115 V, respectively. CID-MS^n experiments were performed on mass-selected precursor ions in the range of *m*/*z* 100–1000. The isolation width of precursor ions was 1.0 mass units. The scan time was equal to 100 ms and the collision energy was optimised between 15-45 (arbitrary units), using helium as collision gas. The data acquisition was carried out by using Xcalibur® data system (ThermoFinnigan, San Jose, CA, USA).

3.6.5.5 HPLC-UV quantification

Calibration curves were obtained by HPLC-UV injection of gallic acid, protocatechuic acid, ellagic acid, quercetin, and naringenin standard solutions in MeOH, with five different concentrations between 5 and 500 µg mL^{-1}. The calibration curves and additional relevant data are shown in Table 3.5.

Table 3.5 – Calibration data used for HPLC-UV quantification of *E. globulus* bark extracts.

Compound	λ (nm)	Conc. Range (µg mL^{-1})	Calibration curve[a]	R^2	LOD (µg mL^{-1})	LOQ (µg mL^{-1})
Gallic acid	280	5 –300	y = 241397x-68195	0.999	10.19	33.95
Protocatechuic acid	280	5 – 500	y = 298281x+1768056	0.999	17.64	58.79
Ellagic acid	340	5 – 400	y = 226944x-792665	1.000	10.76	35.87
Quercetin	340	5 – 250	y = 520780x-449265	0.993	27.78	92.59
Naringenin	280	5 – 200	y = 631833x+152036	0.993	27.74	92.48

[a] y = peak area, x = concentration in µg mL^{-1}

The quantification of individual compounds was accomplished with calibration data for the most similar standard, since for some of them no pure reference compounds were available. Concentrations were calculated in triplicate and the mean value computed in each case.

3.6.5.6 Antioxidant activity

The antioxidant activity of the extracts was determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging methodology [14]. In test tubes, 0.25 ml of

DPPH 0.8 mM in MeOH was added to accurately weighed aliquots of the extracts dissolved in 3.75 ml of MeOH, corresponding to concentration ranges of extract between 0.5 and 8 μg mL^{-1} for MeOH/H_2O and EtOH/H_2O extracts, and between 30 and 350 μg mL^{-1} for supercritical extracts. After mixing, the samples were maintained in the dark, at room temperature for 30 minutes. The absorbance at 517 nm was measured using a UV/Vis V-530 spectrophotometer (Jasco, Tokyo, Japan) and compared with a control without extract. A blank was prepared for each sample using methanol instead of the DPPH solution. Ascorbic acid and 3,5-di-*tert*-4-butylhydroxytoluene (BHT) were used as reference compounds.

The antioxidant activity was expressed as a percent inhibition of DPPH radical, and calculated by:

$$\text{Scavenging activity } (\%) = \frac{\text{Abs control- Abs sample}}{\text{Abs control}} \times 100\% \quad (3.2)$$

IC_{50} values were determined from the plotted graphs of scavenging activity against the concentration of the extracts. These values are defined as inhibitory concentration of the extract necessary to decrease the initial DPPH radical concentration by 50% and are expressed in μg mL^{-1}. Triplicate measurements were carried out. The antioxidant activity was also expressed in g of ascorbic acid equivalents (AAE) per g of bark or per g of extract.

3.7 Results and discussion

3.7.1 Preliminary solid-liquid (SLE) and supercritical fluid (SFE) extractions

3.7.1.1 Extraction yield (EY) and total phenolic content (TPC)

The extraction yield and total phenolic content corresponding to the SLEs carried out with MeOH/H_2O and EtOH/H_2O, and to the SFEs performed with CO_2/EtOH, CO_2/EtOAc, CO_2/H_2O and pure CO_2 are shown in Table 3.6

The EtOH/H_2O extraction yield was slightly higher (9.74%) than that obtained with MeOH/H_2O (9.28%). Moreover, the MeOH/H_2O extraction yield is in accordance with previous results [5]. The SFE yields are considerably lower, varying between 0.04% (CO_2/H_2O) and 0.32% (CO_2/EtOH). The use of ethyl acetate and water as co-solvents lead to very low extraction yields – 0.08% and 0.04%, respectively – in comparison with ethanol.

The total phenolic content of EtOH/H_2O extract (159.57±6.75 mg GAE g^{-1} of extract) is in the range of previously published values for the same biomass [15], and is appreciably lower than that obtained with MeOH/H_2O (407.41±16.68 mg GAE g^{-1} of extract). When supercritical solvents are used, the total phenolic content decreases significantly to values between 9.22±0.27 and 33.10±0.53 mg GAE g^{-1} of extract (Table 3.6). Moreover, the TPC of CO_2/EtOH extract of *E. globulus* bark is in the same range of those already described for supercritical extracts of maritime pine bark at 200-250 bar, 30-50 ºC and 10% ethanol [16].

Table 3.6 – Extraction yield, total phenolic content, and antioxidant activity of solid-liquid and supercritical extracts of *E. globulus* bark.

Extracts	Extraction yield (%)	Total phenolic content (mg GAE g^{-1} of extract)	Antioxidant activity	
			IC_{50} values (µg mL^{-1})	values in AAE[a] (mg AAE g^{-1} of bark)
MeOH/H_2O	9.28	407.41±16.68	3.06±0.09	63.35±1.86
EtOH/H_2O	9.74	159.57±6.75	4.23±0.04	48.13±0.50
CO_2/EtOH	0.32	33.10±0.53	64.81±3.48	0.10±0.01
CO_2/EtOAc	0.08	16.59±0.10	>350	<0.005
CO_2/H_2O	0.04	9.22±0.27	>350	<0.002
CO_2	0.05	10.92±0.23	>350	<0.003

[a] ascorbic acid equivalents

Globally, these results are in agreement with previous studies where low extraction yields of phenolic compounds from guava seeds were obtained with ethyl acetate as CO_2 modifier [17]. Ashraf-Khorassani *et al.* [18] also verified the decrease of phenolic compounds solubility when water is used as co-solvent, whereas better results were reported for several biomass sources using ethanol as co-solvent [10].

3.7.1.2 Antioxidant activity

The antioxidant activity of the solid-liquid and supercritical extracts, expressed as IC_{50} values and as ascorbic acid equivalents, is presented in Table 3.6.

The MeOH/H_2O and EtOH/H_2O extracts revealed a slightly lower DPPH radical scavenging capacity than that of ascorbic acid (measured as control: IC_{50} = 2.09 µg mL^{-1}) and are considerably higher than that of 3,5-di-*tert*-4-butylhydroxytoluene (BHT) (measured as control: IC_{50} = 18.37 µg mL^{-1}) which is commonly observed in extracts from vegetal sources rich in phenolic compounds (e.g., *Eucalyptus* spp. bark and cork from *Quercus suber*) [6, 14]. Furthermore the antioxidant activity of these extracts (in ascorbic acid equivalents) is considerably higher than those reported by Vázquez *et al.* [15] for *E. globulus*, and by Santos *et al.* [6] for other *Eucalyptus* species.

Concerning the SFE extracts, only the antioxidant activity of the CO_2/EtOH extract was possible to measure (IC_{50} = 64.81 μg mL^{-1}) and it was slightly lower than those obtained by SLE. For the remaining supercritical extracts the IC_{50} values were not determined. In fact, it was possible to measure the antioxidant activity for no more than a concentration of 350 μg mL^{-1}, which proves the IC_{50} value is clearly higher than that.

3.7.1.3 Phenolic compounds profiles of the supercritical extracts

Table 3.7 shows the identification and quantification of phenolic compounds detected by HPLC–MS in the supercritical extracts obtained with CO_2 and CO_2/EtOH. The methodology applied is reported elsewhere (Chapter 3-Part A) [5, 6]. Taking into account the poor extraction yields and antioxidant activity results found above for CO_2/EtOAc and CO_2/H_2O, their HPLC–MS analysis was not accomplished. The pure CO_2 extracts were analysed for comparison in order to emphasize the role played by ethanol in the removal of phenolic compounds.

Table 3.7 – HPLC quantification of phenolic compounds identified in supercritical fluid extracts of *E. globulus* bark

No.	Rt (min)	Compound	λ (nm)	Calibration curve (see Table 3.5)	Phenolic content[a] (mg g^{-1} of extract)	
					CO_2/EtOH	CO_2
1	4.0	Gallic acid	280	Gallic acid	0.55	-
2	5.5	Protocatechuic acid	280	Protocatechuic acid	0.54	-
3	15.6	Digalloylglucose	280	Gallic acid	3.84	1.48
4	18.2	Isorhamnetin-hexoside	340	Quercetin	0.26	-
5	18.7	Ellagic acid	340	Ellagic acid	0.66	-
6	20.3	Taxifolin	280	Naringenin	1.73	-
7	22.6	Methyl-ellagic acid-pentoside	340	Ellagic acid	3.06[b(7+8)]	-
8	23.6	Isorhamnetin-rhamnoside	340	Ellagic acid		-
9	25.5	Methyl-ellagic acid	340	Ellagic acid	3.31	-
10	27.2	Mearnsetin	340	Quercetin	Traces	-
11	29.7	Mearnsetin-hexoside	340	Quercetin	Traces	-
12	32.1	Eriodictyol	280	Naringenin	14.75	-
13	33.9	Luteolin	340	Quercetin	1.44[b(13+14)]	-
14	34.0	Quercetin	340	Quercetin		-
15	36.5	Isorhamnetin	340	Quercetin	14.29	-
16	38.4	Naringenin	280	Naringenin	11.38	Traces
Total (mg g^{-1} of extract)					**55.83**	**1.48**
Total (mg kg^{-1} of bark)					**178.67**	**0.74**

[a]Results correspond to the average value estimated from the injection of three aliquots analysed in triplicate (standard deviation <5%). [b]Due to the overlapped peaks, the wavelength where the peak area was higher was chosen.

It is evident from Table 3.7 that ethanol increments significantly the phenolic compounds extraction, as only digalloylglucose **3** and naringenin **16** (Figure 3.13) were detected in the extraction with pure carbon dioxide, while sixteen phenolic compounds were detected by HPLC-MS when modified CO_2 was used in the process. The total concentrations in both cases are also elucidative: 55.83 against 1.48 mg g^{-1} of extract. These values are in accordance with the very distinct phenolic contents and antioxidant activities discussed above (see Table 3.6).

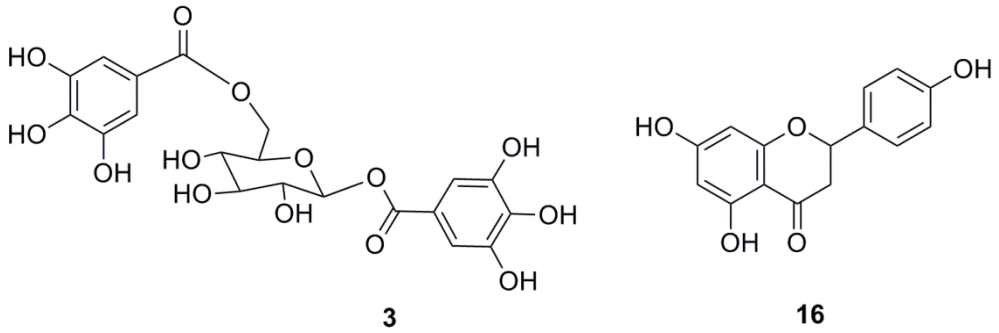


Figure 3.13 – Phenolic compound identified in CO_2 supercritical fluid extraction of *E. globulus* bark

It is worth noting the high contents of flavanones, namely eriodictyol **12** (14.75 mg g^{-1}) and naringenin **16** (11.38 mg g^{-1}), and the *O*-methylated flavonol isorhamnetin **15** (14.29 mg g^{-1}) (Figure 3.14), when compared with the contents previously achieved with conventional MeOH/H_2O SLE [5]. In fact, the high content of flavanones in supercritical extracts, as for example from root bark from osage orange tree, has already been reported before [19].

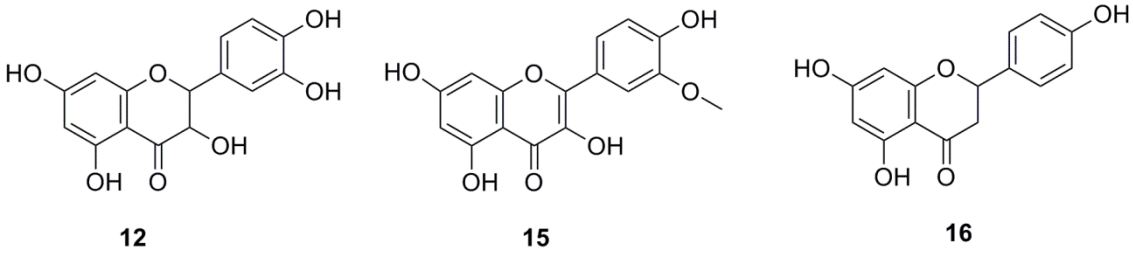


Figure 3.14 – Major phenolic compounds identified in CO_2/EtOH supercritical fluid extraction of *E. globulus* bark

Finally, it should be emphasized that the phenolic composition of a supercritical *E. globulus* bark extract analysed by HPLC-MS is published here for the first time.

3.7.2 Analysis of the designed SFE experiments

According to the results discussed above, carbon dioxide modified with ethanol is the most suitable solvent for the supercritical extraction of phenolic compounds of *E. globulus* bark. In this work, the influence of temperature, ethanol content, and CO_2 flow rate were analysed in order to optimise the extraction conditions. Pressure was fixed at the highest value achieved by our apparatus (300 bar), which is well inside the intervals found in the literature for similar studies [10]. However, it is well known that the influence of pressure is positive upon phenolic compounds removal [17], as also several studies have verified the increase of the phenolic compounds solubility with the increase of pressure [20, 21], and thus it is of interest to evaluate the remaining operating parameters.

The full factorial design adopted and the experimental results obtained are listed in Table 3.8 namely: extraction yield (EY), total phenolic content (TPC), total amount of phenolic compounds quantified by HPLC (PC-HPLC), and antioxidant activity (AA) expressed as ascorbic acid equivalents.

Table 3.8 – Coded and uncoded levels of independent variables for the 2^3 full factorial design and experimental values obtained for response variables

Exp.	A	B	C	*T* (ºC)	% of EtOH	Q_{CO_2} (g min^{-1})	EY (%)	TPC (mg GAE g^{-1} of extract)	PC-HPLC (mg g^{-1} of extract)	AA (mg AAE g^{-1} of extract)
1	-1	1	-1	50	20	8	0.39	44.65	127.28	29.38
2	1	1	1	70	20	10	0.48	61.20	125.76	57.72
3	1	-1	-1	70	15	8	0.32	33.10	55.83	32.31
4	1	1	-1	70	20	8	0.51	37.98	78.98	36.26
5	-1	1	1	50	20	10	0.34	41.98	92.22	30.87
6	-1	-1	1	50	15	10	0.28	30.29	65.04	16.39
7	-1	-1	-1	50	15	8	0.28	36.92	73.53	15.98
8	1	-1	1	70	15	10	0.29	44.53	89.33	39.55
9	0	0	0	60	17.5	9	0.42	37.16	84.87	39.43
10	0	0	0	60	17.5	9	0.39	38.42	77.49	41.72
11	0	0	0	60	17.5	9	0.37	39.93	80.28	38.07

The regressed coefficients of equation (3.1) are listed in Table 3.9. It is important to emphasize that the final models presented in the following sections only contain statistically significant coefficients (90% confidence level), notwithstanding, the recalculated surfaces provide worse correlation coefficients.

Table 3.9 – Parameters of the full regression model (Equation (3.1)) for the SFE factorial design presented in Table 3.8

Coefficient	Variable	EY (%)	TPC (mg GAE g^{-1} of extract	PC-HPLC (mg g^{-1} of extract)	AA (mg AAE g^{-1} of extract)
b_0		0.3700*	40.5600*	86.4191*	34.334*
b_1	Temperature	0.0388*	2.8712**	-1.0212	9.1525*
b_2	% of EtOH	0.0688*	5.1212*	17.5638*	6.2500**
b_3	CO_2 flow rate	-0.0138	3.1688*	4.5912	3.8250
b_{12}		0.0262	0.2662	-2.6687	-0.7200
b_{13}		-0.0012	5.4938*	15.4788*	3.3500
b_{23}		-0.0062	1.9688	-1.6612	1.9125
b_{123}		0.0062	0.9788	4.9812	1.6425
R^2		0.942	0.968	0.969	0.907

* Significant at p≤0.05; ** Significant at p≤0.10

3.7.2.1 Influence of process variables on SFE yield

The SFE yield of *E. globulus* bark has shown to be positively affected by ethanol content (p≤0.05) and temperature (p≤0.05), while the effect of CO_2 flow rate and the interactions between variables were not significant (Table 3.9). The resulting linear regression model is:

$$EY\ (\%)=0.370+0.0388T+0.0688EtOH \quad (3.3)$$

and it is plotted in Figure 3.13; the adjusted correlation coefficient is R^2_{adjust}=0.817. An extraction yield of 0.48% may be achieved at the highest temperature (T = 70 ºC) and ethanol content (20%). Despite these yields are slightly lower than those achieved in the SFE of phenolic fractions from vegetal sources, like roasted wheat germ and maritime pine bark [16, 22], it has to be noted that the bark samples of this study were previously submitted to a Soxhlet dichloromethane extraction, which removed undesired less polar compounds and consequently decreased the SFE yields.

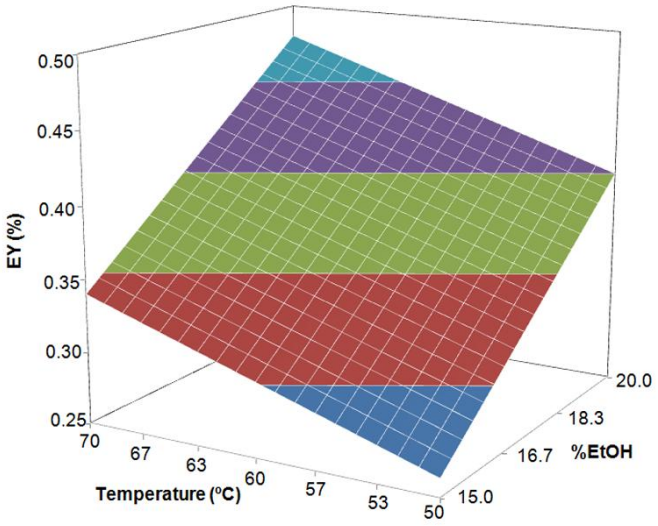


Figure 3.13 – Influence of temperature and ethanol content on the SFE yield at constant solvent flow rate (Q_{CO2} = 10 g min^{-1}).

3.7.2.2 Influence of process variables on total phenolic content (TPC)

The regression analysis of experimental data showed that the TPC was significantly ($p \leq 0.05$) affected by ethanol content and CO_2 flow rate (Table 3.9). The ethanol content in the supercritical solvent has been repeatedly described as a positive factor upon the TPC of vegetal and fruits extracts measured the by Folin-Ciocalteu method [23, 24], since it increases the solvating power of CO_2. On the other hand the influence of CO_2 flow rate is strictly related with external mass transfer phenomena [8]. The effect of temperature and its interaction with CO_2 flow rate were also significant ($p<0.10$), while the other interactions were not (Table 3.9). The final regression model is given by:

$$\text{TPC (mg GAE.g}^{-1}\text{ of extract)} = 40.6 + 2.87T + 5.12EtOH + 3.17Q + 5.49TQ \quad (3.4)$$

for which the calculated R^2_{adjust} is 0.908. From the fitted surface, graphically shown in Figure 3.14, it is clear that an increase in either ethanol content or solvent flow rate, at constant temperature, favours TPC.

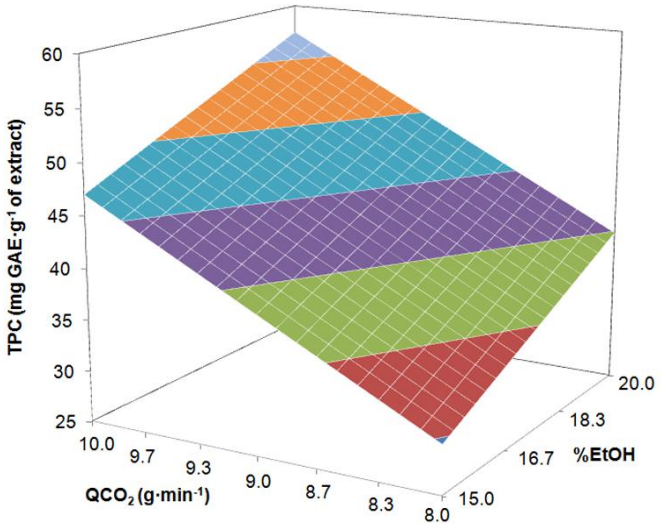


Figure 3.14 – Influence of solvent flow rate and ethanol content on the total phenolic content of the supercritical extracts at 70 ºC

A maximum value of TPC may be achieved (57.22 mg g^{-1}) for the highest values of the process parameters under study. This value is slightly higher than those already published for the optimised SFE of phenolic compounds from vegetal sources like roasted wheat germ (*P* = 336 bar, *T* = 58 ºC) [22] and strawberry fruits (*P* = 60 bar, *T* = 48 ºC) [23].

3.7.2.3 Influence of process variables on total amounts of phenolic compounds quantified by HPLC

The total amounts of phenolic compounds quantified by HPLC (PC-HPLC) depend significantly upon ethanol content. Murga *et al.* [25] observed that the solubilities of phenolic compounds like gallic acid, catechin, and epicatechin increase with increasing modifier concentration. This influence is related with covalent (hydrogen bonding) and dipole-dipole interactions between co-solvent and polar solutes. For instance, the solubility of quercetin enhances with the increase of ethanol content due to alcohol-phenol interactions [21]. Even though not statistically significant, and contrary to the other response factors, the SFE temperature imparts a negative influence on the total phenolic compounds quantified by HPLC (see Table 3.9, coefficient = -1.02), which means that its positive effect upon the vapour pressure of these solutes does not overcome the resulting solvent density reduction. Consequently, solubility diminishes [26]. Although this reasoning could imply that the operating pressure is behind the cross-over value, the influence of temperature on the phenolic composition may be due to another aspect: the degradation of phenolic compounds, which may take place above 50 ºC [10]. It is worth noting that the negative influence of temperature was not

detected in all response factors, which may be related with the fact that the phenolic compounds identified/quantified by HPLC and those measured by reduction with the Folin-Ciocalteu reagent are not exactly the same.

The regression analysis of the PC-HPLC data gave rise to the following quadratic model:

$$\text{PC-HPLC (mg.g}^{-1}\text{ of extract)}=86.4+17.6\text{EtOH}+15.5\text{TQ} \quad (3.5)$$

in which the response is linearly dependent on the ethanol content and on the temperature-CO_2 flow rate interaction, with R^2_{adjust} = 0.878. The fitted surface is plotted in Figure 3.15, whose maximum reaches 119.46 mg g^{-1} of extract at 70 ºC, 20% of ethanol, and CO_2 flow rate of 10 g min^{-1}.

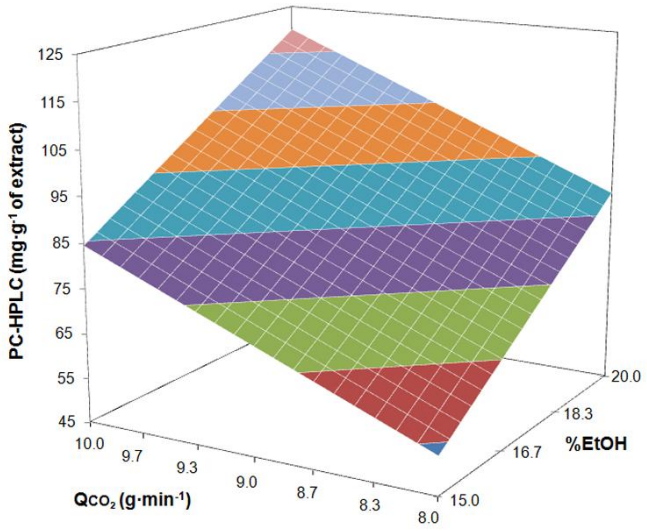


Figure 3.15 – Influence of ethanol content and solvent flow rate on the phenolic content measured by HPLC at 70 ºC

3.7.2.4 Influence of process variables on antioxidant activity

Temperature was the most influential variable (p≤0.05) for the antioxidant activity, followed by the ethanol content (p≤0.10), whereas CO_2 flow rate and the interactions between variables were not statistically significant (p>0.05) (Table 3.9). This is in accordance with published results on the influence of these process parameters on the antioxidant activity of extracts from several biomass sources [10]. Hence, the model fitted to the experimental data is given by:

$$\text{AA (mg AAE.g}^{-1}\text{of extract)}=34.3+9.15\text{T}+6.25\text{EtOH} \quad (3.6)$$

with R^2_{adjust} value of 0.716. From this surface, represented graphically in Figure 3.16, the maximum antioxidant activity is 49.74 mg AAE g^{-1} of extract, corresponding to a IC_{50} value of 42.02 µg mL^{-1} by setting the temperature at 70 ºC and the ethanol content at 20%. This value is distant to those achieved above with conventional EtOH/H_2O and MeOH/H_2O extractions (4.23 and 3.06 µg mL^{-1}, respectively), due to the high supercritical selectivity for flavanones achieved under the experimental conditions studied. As has been mentioned above, methanolic extracts of *E. globulus* bark provided 29 phenolic compounds [5], while only 15 have been quantified in this work after SFE. Nonetheless, one may emphasize that our result is quite better in comparison to that obtained for oregano extracts when supercritical carbon dioxide was utilised with ethanol also [27].

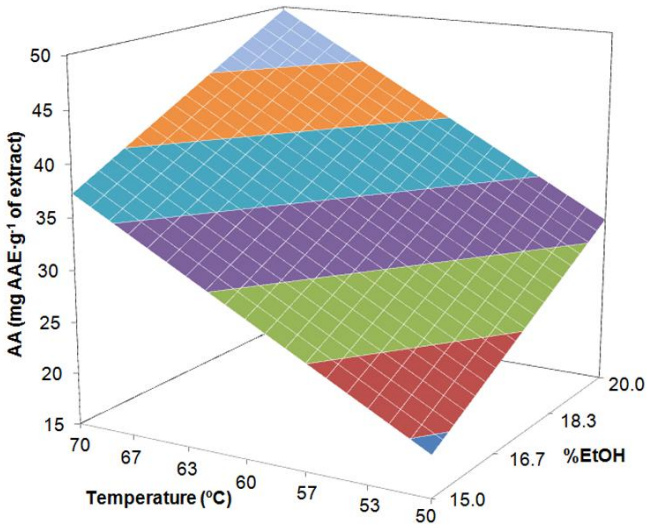


Figure 3.16 – Influence of ethanol content and temperature on the antioxidant activity at constant solvent flow rate (Q_{CO2} = 10 g min^{-1})

3.7.2.5 Optimisation of supercritical fluid extraction conditions

The best operating conditions for the SFE of phenolic compounds from *E. globulus* bark were determined in order to obtain the maximum quality of the extract, i.e. the maximum values of the response factors under investigation (EY, TPC, PC-HPLC, AA). The optimised temperature, ethanol content, and CO_2 flow rate were found to be 70 ºC, 20% and 10 g min^{-1}, respectively. The predicted results computed with the reduced regression models (i.e., containing only statistically significant factors) are: EY = 0.48%, TPC = 57.22 mg GAE g^{-1} of extract, PC-HPLC = 119.46 mg g^{-1} of extract, and AA = 49.74 mg AAE g^{-1} of extract. These predictions match very well with the experimental results achieved at this point: EY = 0.48%, TPC = 61.20 mg GAE g^{-1} of

extract, PC-HPLC = 125.76 mg g^{-1} of extract, and AA = 57.72 mg AAE g^{-1} of extract. Such fact validates the models proposed in this study to predict the response factors in the range of the experimental conditions covered.

3.8 Conclusions

The supercritical fluid extraction of phenolic compounds from *E. globulus* bark, using pure and modified CO_2 with water, ethyl acetate, and ethanol, was analysed in this work for the first time. The phenolic compounds profiles of the extracts were determined by HPLC-MS and reported here for the first time also. The best preliminary results in terms of selectivity towards phenolic components, extraction yield, and antioxidant activity were achieved with CO_2/EtOH. Furthermore this extract contained much higher quantities of eriodictyol and naringenin (flavanones), and isorhamentin (*O*-methylated flavonol) than the conventional solid-liquid extracts obtained previously with methanol/water mixture.

A full 2^3 design of experiments was carried out to investigate in detail the effects of temperature, ethanol content, and CO_2 flow rate upon the supercritical extraction yield (EY), total phenolic content (TPC), phenolic compounds quantified by HPLC (PC-HPLC), and antioxidant activity (AA) of extracts. Under the experimental conditions tested, the main results were: the ethanol content imparted a significant and positive influence in the four responses, the temperature did not affect only the phenolic content quantified by HPLC, and the CO_2 flow rate only had influence on total phenolic content. In the whole, the conditions maximizing all responses were found as 70 ºC, 20 wt% of ethanol, and 10 g of CO_2 min^{-1} at 300 bar. The values of the dependent variables at this point were: EY = 0.48% of extraction yield, TPC = 57.22 mg GAE g^{-1} of extract, PC-HPLC = 119.46 mg g^{-1} of extract, and AA = 49.74 mg AAE g^{-1} of extract.

References

[1] Quilhó, T.; Pereira, H., Within and between-tree variation of bark content and wood density of *Eucalyptus globulus* in commercial plantations. *IAWA J.* **2001,** *22*, 255-265.

[2] Freire, C. S. R.; Silvestre, A. J. D.; Neto, C. P.; Cavaleiro, J. A. S., Lipophilic extractives of the inner and outer barks of *Eucalyptus globulus*. *Holzforschung* **2002,** *56*, 372-379.

[3] Domingues, R. M. A.; Sousa, G. D. A.; Freire, C. S. R.; Silvestre, A. J. D.; Neto, C. P., *Eucalyptus globulus* biomass residues from pulping industry as a source of high value triterpenic compounds. *Ind. Crop Prod.* **2010,** *31*, 65-70.

[4] Manach, C.; Scalbert, A.; Morand, C.; Remesy, C.; Jimenez, L., Polyphenols: food sources and bioavailability. *Am. J. Clin. Nutr.* **2004,** *79*, 727-747.

[5] Santos, S. A. O.; Freire, C. S. R.; Domingues, M. R. M.; Silvestre, A. J. D.; Neto, C. P., Characterization of Phenolic Components in Polar Extracts of *Eucalyptus globulus* Labill. Bark by High-Performance Liquid Chromatography-Mass Spectrometry. *J. Agric. Food Chem.* **2011,** *59*, 9386-9393.

[6] Santos, S. A. O.; Villaverde, J. J.; Freire, C. S. R.; Domingues, M. R. M.; Neto, C. P.; Silvestre, A. J. D., Phenolic composition and antioxidant activity of *Eucalyptus grandis*, *E. urograndis* (*E. grandis* x *E. urophylla*) and *E. maidenii* bark extracts. *Ind. Crop Prod.* **2012,** *39*, 120-127.

[7] Hauthal, W. H., Advances with supercritical fluids [review]. *Chemosphere* **2001,** *43*, 123-135.

[8] Reverchon, E.; De Marco, I., Supercritical fluid extraction and fractionation of natural matter. *J. Supercrit. Fluid* **2006,** *38*, 146-166.

[9] Lang, Q.; Wai, C. M., Supercritical fluid extraction in herbal and natural product studies - a practical review. *Talanta* **2001,** *53*, 771-782.

[10] Díaz-Reinoso, B.; Moure, A.; Domínguez, H.; Parajó, J. C., Supercritical CO_2 extraction and purification of compounds with antioxidant activity. *J. Agric. Food Chem.* **2006,** *54*, 2441-2469.

[11] Abdulagatov, A.; Stepanov, G.; Abdulagatov, I., The critical properties of binary mixtures containing carbon dioxide: Experimental data. *High Temp.+* **2007,** *45*, 85-126.

[12] Yeo, S.-D.; Park, S.-J.; Kim, J.-W.; Kim, J.-C., Critical Properties of Carbon Dioxide + Methanol, + Ethanol, + 1-Propanol, and + 1-Butanol. *J. Chem. Eng. Data* **2000,** *45*, 932-935.

[13] Falco, N.; Kiran, E., Volumetric properties of ethyl acetate+carbon dioxide binary fluid mixtures at high pressures. *J. Supercrit. Fluid* **2012,** *61*, 9-24.

[14] Santos, S. A. O.; Pinto, P. C. R. O.; Silvestre, A. J. D.; Neto, C. P., Chemical composition and antioxidant activity of phenolic extracts of cork from *Quercus suber* L. *Ind. Crop Prod.* **2010,** *31*, 521-526.

[15] Vázquez, G.; Fontenla, E.; Santos, J.; Freire, M. S.; González-Álvarez, J.; Antorrena, G., Antioxidant activity and phenolic content of chestnut (*Castanea sativa*) shell and eucalyptus (*Eucalyptus globulus*) bark extracts. *Ind. Crop. Prod.* **2008,** *28*, 279-285.

[16] Seabra, I. J.; Dias, A. M. A.; Braga, M. E. M.; de Sousa, H. C., High pressure solvent extraction of maritime pine bark: Study of fractionation, solvent flow rate and solvent composition. *J. Supercrit. Fluid* **2012,** *62*, 135-148.

[17] Castro-Vargas, H. I.; Rodríguez-Varela, L. I.; Ferreira, S. R. S.; Parada-Alfonso, F., Extraction of phenolic fraction from guava seeds (*Psidium guajava* L.) using supercritical carbon dioxide and co-solvents. *J. Supercrit. Fluid* **2010,** *51*, 319-324.

[18] Ashraf-Khorassani, M.; Gidanian, S.; Yamini, Y., Effect of Pressure, Temperature, Modifier, Modifier Concentration, and Sample Matrix on the Supercritical Fluid Extraction Efficiency of Different Phenolic Compounds. *J. Chromatogr. Sci.* **1995,** *33*, 658-662.

[19] da Costa, C. T.; Margolis, S. A.; Benner Jr, B. A.; Horton, D., Comparison of methods for extraction of flavanones and xanthones from the root bark of the osage orange tree using liquid chromatography. *J. Chromatogr. A* **1999,** *831*, 167-178.

[20] Murga, R.; Sanz, M. a. T.; Beltrán, S.; Cabezas, J. L., Solubility of some phenolic compounds contained in grape seeds, in supercritical carbon dioxide. *J. Supercrit. Fluid* **2002,** *23*, 113-121.

[21] Chafer, A.; Fornari, T.; Berna, A.; Stateva, R. P., Solubility of quercetin in supercritical CO_2 plus ethanol as a modifier: measurements and thermodynamic modelling. *J. Supercrit. Fluids* **2004,** *32*, 89-96.

[22] Gelmez, N.; Kincal, N. S.; Yener, M. E., Optimization of supercritical carbon dioxide extraction of antioxidants from roasted wheat germ based on yield, total phenolic and tocopherol contents, and antioxidant activities of the extracts. *J. Supercrit. Fluids* **2009,** *48*, 217-224.

[23] Akay, S.; Alpak, I.; Yesil-Celiktas, O., Effects of process parameters on supercritical CO_2 extraction of total phenols from strawberry (*Arbutus unedo* L.) fruits: An optimization study. *J. Sep. Sci.* **2011,** *34*, 1925-1931.

[24] Adil, I. H.; Çetin, H. I.; Yener, M. E.; Bayindirli, A., Subcritical (carbon dioxide + ethanol) extraction of polyphenols from apple and peach pomaces, and determination of the antioxidant activities of the extracts. *J. Supercrit. Fluid* **2007,** *43*, 55-63.

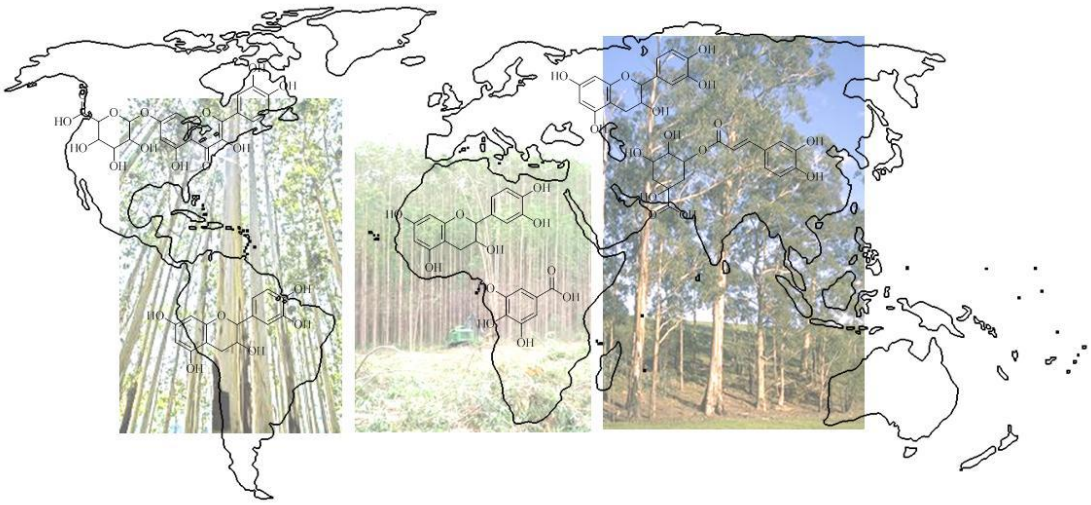
[25] Murga, R.; Ruiz, R.; Beltran, S.; Cabezas, J. L., Extraction of natural complex phenols and tannins from grape seeds by using supercritical mixtures of carbon dioxide and alcohol. *J. Agric. Food Chem.* **2000,** *48*, 3408-3412.

[26] Uchiyama, H.; Mishima, K.; Oka, S.; Ezawa, M.; Ide, M.; Takai, T.; Park, P. W., Solubilities of flavone and 3-hydroxyflavone in supercritical carbon dioxide. *J. Chem. Eng. Data* **1997,** *42*, 570-573.

[27] Cavero, S.; Garcia-Risco, M. R.; Marin, F. R.; Jaime, L.; Santoyo, S.; Senorans, F. J.; Reglero, G.; Ibanez, E., Supercritical fluid extraction of antioxidant compounds from oregano - Chemical and functional characterization via LC-MS and in vitro assays. *J. Supercrit. Fluids* **2006,** *38*, 62-69.

Chapter 4

Characterisation of phenolic fraction of *Eucalyptus grandis*, *E. urograndis* (*E. grandis* ×*E. urophylla*) and *E. maidenii* barks



Adapted from:

Santos, S.A.O., Villaverde, J.J., Freire, C.S.R., Domingues, M.R.M., Neto, C.P., Silvestre, A.J.D., Phenolic composition and antioxidant activity of *Eucalyptus grandis*, *E. urograndis* (*E. grandis* x *E. urophylla*) and *E. maidenii*, *Industrial Crops and Products*, **2012**, 39, 120-127

Abstract

The phenolic composition of *E. grandis*, *E. urograndis (E. grandis* x *E. urophylla)* and *E. maidenii* bark is reported for the first time. High-performance liquid chromatography–electrospray ionization mass spectrometry (HPLC–ESI–MS) and multi-stage mass spectrometry (MS^n) analysis of the methanol:water (50:50) extracts allowed to identify thirteen, twelve and twenty four phenolic compounds in *E. grandis*, *E. urograndis* and *E. maidenii* bark extracts, respectively. Furthermore, ellagic acid-rhamnoside, dihydroxy-isopropylchromone-hexoside and dihydroxy-(methylpropyl)isopropylchromone-hexoside are referenced for the first time as constituents of *Eucalyptus* species. Epicatechin and quercetin-glucuronide are the major phenolic compounds in *E. grandis* and *E. urograndis* bark, followed by ellagic acid-rhamnoside and ellagic acid in *E. grandis* and by galloyl-bis-hexahydroxydiphenoyl (HHDP)-glucose and gallic acid in *E. urograndis*. Catechin, chlorogenic acid and methyl-ellagic acid-pentoside are the major compounds in *E. maidenii* bark. The phenolic content of the three extracts shows a positive correlation with their antioxidant activities, evaluated by 2,2-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging, showing activity values between those of two commercial antioxidants, ascorbic acid and butylated hydroxytoluene (BHT). These results, together with the phenolic composition, confirm the high potential of these species as source of biologically active phenolic compounds.

4.1 Introduction

Eucalyptus species are the main wood sources for pulp and paper production worldwide, due to their fast growing and short rotation periods as well as to favourable pulping and bleaching ability [1]. *Eucalyptus* plantations area cover around 19 million hectares worldwide [2], with *E. grandis* as the most cultivated specie for industrial purposes, particularly in South Africa and Brazil [1]. *E. urograndis*, an hybrid between *E. grandis* and *E. urophylla,* is produced in Brazil and was developed to conjugate the fast growing properties of *E. grandis* and the high density and superior pulp properties of *E. urophylla* wood [3], demonstrating the increasing interest on the exploitation of *Eucalyptus* spp. to pulp and paper production in the South America. In fact, in the last five years the eucalyptus planted area in Brazil has increased 5.3 % per year, being the 6th world pulp producer in 2010 [4]. *E. maidenii* is presently not so widely used as a fiber source for pulp production as others *Eucalyptus* species, nevertheless its potential for forest developing and excellent pulp qualities has also been demonstrated [5].

It is well known that pulp and paper industries generate high amounts of residues, including mostly bark, but also leaves, branches, fruits and knots, which are commonly burned on the biomass boilers or just leaved on the forest for fertilization purposes. However, in the last years, these by-products, as many other agro-forest residues, are seen as promising sources of materials, chemicals, fuels or energy, to take their maximum value out as also a response of the depletion of fossil resources, within the biorefinery concept [6-8]. In fact, the exploitation of agro-forest residues as a source of valuable compounds is a strategy already applied in some pulp mills and one of the most popular examples of the implementation of the biorefinery concept [9, 10].

In this perspective, in recent years, the biomass residues of the exploitation *Eucalyptus* species, and particularly outer bark, have attracted much interest namely as sources of high value triterpenic acids [11-13]. More recently, *E. globulus* bark was studied as source of phenolic compounds [14]. However, the information of the phenolic composition of *E. grandis*, *E. urograndis* and *E. maidenii* is scarce and only studies about the presence of these compounds on the leaf litter from *E. urograndis* [15] and leaves from *E. grandis* [16] and *E. maidenii* [17] have been published. To the best of our knowledge, no study has been carried out about the phenolic composition of the barks from these species, despite the well-known wide range of valuable properties assigned to phenolic compounds, such as anti-inflammatory, antioxidant, antibacterial,

antimicrobial, antithrombotic or even anticarcinogenic and anti-HIV-1 agents, among others [18, 19].

In this context, and following our interest on the study of the chemical composition and valorisation of *Eucalyptus* barks, the aim of this study is to evaluate the potential of *E. grandis*, *E. urograndis* and *E. maidenii* bark as sources of valuable phenolic compounds, analyzing their methanol:water extracts by high-performance liquid chromatography-mass spectrometry (HPLC-MS) and also accessing their total phenolic content by the Folin-Ciocalteu assay and their antioxidant properties determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging.

4.2 Materials and methods

4.2.1 Chemicals

Dichloromethane (99% purity), gallic acid (purity higher than 97.5%) and quercetin (purity higher than 98%) were supplied by Sigma Chemical Co (Madrid, Spain). Protocatechuic acid (purity higher than 97%), chlorogenic acid (purity higher than 95%) and naringenin (98% purity) were obtained from Aldrich Chemical Co (Madrid, Spain). HPLC-grade methanol, water and acetonitrile, were supplied from Fisher Scientific Chemicals (Loures, Portugal). Formic acid (purity higher than 98%), methanol (purity higher than 99.8%), catechin (purity higher than 96%) and ellagic acid (96% purity) were purchased from Fluka Chemie (Madrid, Spain). Solvents were filtered using a Solvent Filtration Apparatus 58061 from Supelco (Bellefonte, PA, USA).

4.2.2 Raw materials

E. urograndis and *E. grandis* bark samples were taken from a 5-year-old and 10-year-old tree, respectively, randomly harvested from clone plantations cultivated in Alfredo Chaves, state of Espírito Santo, Brazil (20º38’08’’S, 40º44’57’’W), while *E. maidenii* bark was obtained from a 10-year-old tree, randomly sampled in a clone plantation cultivated in Odemira, southwestern region of Portugal (37º33’04’’N, 8º38’43’’W).

4.2.3 Phenolic compounds extraction

Eucalyptus bark of each species was air dried, until a constant weight was achieved, and ground to granulometry lower than 2 mm prior to extraction.

About 45 g of each dried bark were submitted to a soxhlet extraction with dichloromethane for 6 hours, to remove the lipophilic components. The solid bark

residues were then suspended (m/v: 1:100) in a methanol:water (MeOH:H_2O) mixture, 50/50 (v/v), at room temperature for 24 hours, under constant stirring. The suspensions were then filtered, MeOH removed by low pressure evaporation and the extracts freeze dried [14].

4.2.4 Total phenolic content

The total phenolic content (TPC) of the extracts was determined by the Folin-Ciocalteu method [20, 21]. 2.5 ml of Folin-Ciocalteu reagent, previously diluted with water (1:10, v/v), and 2 ml of aqueous sodium carbonate (75 g L^{-1}) were added to accurately weighed aliquots of the extracts dissolved in 0.5 mL of methanol, corresponding to concentration ranges between 80 and 200 µg of extract mL^{-1}. Each mixture was kept for 5 min at 50 ºC and, after cooling, the absorbance was measured at 760 nm, using a UV–Vis V-530 spectrophotometer (Jasco, Tokyo, Japan). TPC was calculated as gallic acid equivalent from the calibration curve of gallic acid standard solutions (10 - 85.0 µg mL^{-1}) and expressed as g of gallic acid equivalent (GAE) g^{-1} of extract. The analyses were carried out in triplicate and the average value was calculated in each case.

4.2.5 HPLC-UV procedure

Analyses were carried according to a previously reported procedure [14], by using a Hewlett–Packard (HP) 1050 liquid chromatograph (Agilent Technologies, Waldbronn, Germany) equipped with a Rheodyne injector with a 10 µL loop, a quaternary pumping system and a UV detector. It was used a Discovery® C-18 (15 cm x 2.1 mm x 5 µm) column supplied by Supelco (Agilent Technologies, Waldbronn, Germany). The separation of the compounds was carried out with a gradient elution program at a flow rate of 0.2 mL min^{-1}, at room temperature. The mobile phases consisted in water:acetonitrile (90:10, v/v) (A) and acetonitrile (B), both with 0.1% of formic acid. The following linear gradient was applied: 0 - 3min: 0%B; 3-10 min: 0 -10% B; 10 -30 min: 10 - 20% B; 30 - 35 min: 20 - 25%B; 35 – 50 min: 25 - 50% B; 50 – 60 min: 50 - 0% B; followed by re-equilibration of the column for 10 minutes before the next run. The injection volume in the HPLC system was 25 µL and the UV–Vis detection was performed at 280 and 340 nm. Before the injection in the HPLC each extract was dissolved in MeOH:H_2O (50:50), HPLC grade, to obtain a final concentration of about 10 mg mL^{-1} and then filtered through a 0.2 µm PTFE syringe filter.

4.2.6 ESI–QqQ–MS analysis

The HPLC system was coupled to a Micromass spectrometer (Manchester, UK), operating in negative mode, equipped with an electrospray source and a triple quadrupole (QqQ–MS) analyser. The cone and capillary voltages were set at -30.0 V and -2.6 kV, respectively. The source temperature was 143 ^{o}C and the desolvation temperature was 350 ^{o}C. MS/MS spectra were obtained using argon as collision gas with the collision energy set between 10 and 45 V. The detection was carried out considering a mass range of *m*/*z* between 50–1000, with a scan duration of 0.5 s. The data acquisition was done by using the MassLynx® data system (Waters, Milford, MA, U.S.A.).

4.2.7 ESI–IT–MS/MS analysis

To gather additional MS information about several chromatographic peaks, these were manually collected following the chromatographic conditions discussed above. The resulting HPLC fractions were dissolved in methanol and directly injected into a Linear Ion trap LXQ (ThermoFinnigan, San Jose, CA, USA), also equipped with an ESI source by means of a syringe pump, at flow rate of 8 µL min^{-1}. The nitrogen sheath gas was at 30 psi, spray voltage 4.7 kV and capillary temperature 275°C. The capillary and tune lens voltages were set at -7.0 V and -71.8 V, respectively. CID-MS/MS and MS^{n} experiments were performed on mass-selected precursor ions using standard isolation and excitation configuration. The collision energy used was between 15-40 (arbitrary units). The data acquisition was carried out with Xcalibur® data system (ThermoFinnigan, San Jose, CA, USA).

4.2.8 HPLC-UV quantification

Calibration curves were obtained by HPLC injection of gallic acid, protocatechuic acid, catechin, chlorogenic acid, ellagic acid, quercetin and naringenin standard solutions in MeOH, with five different concentrations between 0.01 and 1.20 mg mL^{-1}. The data relevant for obtaining the calibration curves is shown in Table 4.1. Quantification of individual compounds (Table 4.4) was obtained using the calibration data of the most similar standard, as for some of which no pure reference compounds were available. Concentrations were calculated in triplicate and the mean value calculated in each case.

Table 4.1 – Calibration data used for the HPLC-UV quantification of phenolic components of *E. grandis*, *E. urograndis* and *E. maidenii* bark extracts

Compound	λ (nm)	Conc. Range (mg mL^{-1})	Calibration curve[a]	R^2	LOD[b]	LOQ [b]
Gallic acid	280	0.01 – 0.60	y = 535985x+1092	0.999	0.021	0.069
Protocatechuic acid	280	0.05 – 1.20	y = 527991x-5289	1.000	0.029	0.096
Catechin	280	0.01 – 1.20	y = 100865x+2057	1.000	0.028	0.092
Chlorogenic acid	280	0.01 – 0.80	y = 229162x-1450	0.999	0.029	0.097
Ellagic acid	340	0.01 – 0.44	y = 300168x+3045	0.998	0.023	0.076
Quercetin	340	0.01 – 0.33	y = 619494x+2454	0.999	0.014	0.046
Naringenin	280	0.01 – 0.28	y = 722267x+1939	0.998	0.016	0.052

[a] y = peak area, x = concentration in mg mL^{-1}; [b] expressed in mg mL^{-1}

4.2.9 Antioxidant activity

The antioxidant activity of the extracts was determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging [21, 22], as described before (Chapter 3) The concentration ranges of extracts were between 2 and 13 µg mL^{-1}. Ascorbic acid and 3,5-Di-*tert*-4-butylhydroxytoluene (BHT) were used as reference compounds.

Antioxidant activity was expressed as a percent inhibition of DPPH radical, and calculated from the equation described before (equation (3.2)). Triplicate measurements were carried out. The antioxidant activity was expressed as IC_{50} values in µg mL^{-1}, as also in g of ascorbic acid equivalents kg^{-1} of bark (g AAE Kg^{-1} of bark).

4.3 Results and discussion

4.3.1 Extraction yield and total phenolic content

The extraction yields of the MeOH:H_2O bark extracts of the three species analysed and the respective total phenolic content, determined by Folin-Ciocalteu method are shown in Table 4.2. The extraction yields of the studied extracts are distinct between them, with *E. urograndis* showing the higher value (15.18 %).

Table 4.2 – Extraction yields and total phenolic content of *E. grandis*, *E. urograndis* and *E. maidenii* bark extracts

Extracts	Extraction yield (%)	Total phenolic content (mg GAE[a] g^{-1} of extract)	Total phenolic content (mg GAE[a] g^{-1} of bark)
E. grandis	10.54	385.63±11.02	40.64±1.16
E. urograndis	15.18	346.72±7.76	56.92±1.18
E. maidenii	13.23	203.86±4.37	26.97±0.58

Values of total phenolic content are expressed as mean±standard deviation (n=3); [a] GAE: gallic acid equivalents

The total phenolic contents accounted for 385.63±11.02, 346.72±7.76 and 203.86±4.37 mg GAE g^{-1} in *E. grandis*, *E. urograndis* and *E. maidenii* bark extracts, respectively, demonstrating that *E. maidenii* is the species with lower TPC and *E. grandis* and *E. urograndis* extracts have similar TPC contents.

Interestingly although the extraction yields reported for the barks of these three species are higher than those reported earlier for *E. globulus* bark, the total phenolic contents are lower [14, 23].

4.3.2 Identification of phenolic compounds

The identification of the components was carried out by HPLC-UV, HPLC-MS/MS and in some cases by MS^n. Compounds were identified comparing their spectral data with reference compounds, when available, or corroborated with the literature. HPLC-MS data for the identified compounds, namely, their retention time, the molecular ion $[M-H]^-$ and the main product ions obtained by HPLC-MS/MS and by MS^n are given in Table 4.3.

Table 4.3 – Phenolic compounds identified in *E. grandis*, *E. urograndis* and *E. maidenii* bark extracts and corresponding MS/MS and MS^n fragmentation profiles

No.	Rt (min)	Compound	$[M-H]^-$ (*m/z*)	QqQ–MS/MS product ions (*m/z*)	IT–MS^n product ions (*m/z*)	Identified	Presence in *Eucalyptus* species[a]
1	3.2	Quinic acid	191	173, 111, 87, 85	MS^2: 173, 149, 127, 111, 93, 85	[14]	Egl b [14]
2	3.8	Gallic acid	169	125	-	Co[b]	Eur l [15], Egl b [14], w, l, f, Eca Eru l, Ere b, w [24]
3	5.7	Protocatechuic acid	153	109	-	Co	Eur l [15], Egl b [14] Egl Eca Eru b [25],l [26]
4	11.2	Methyl gallate	183	168, 124	-	[14]	Egl b [14], [27]
5	12.5	Catechin	289	245, 205, 203, 125, 109	-	Co	Egl l [28], b [14], [27, 29], w [30], Ere b, w [30]
6	13.6	Chlorogenic acid	353	191	-	Co	Eur l [15], Egl b [14], w [30] Ecy l [31]
7	14.3	Galloyl-bis-HHDP-glucose	935	935, 633, 301, 300, 275	MS^2: **633**[c]; MS^3: 481, 299, 275	[14, 24]	Egl b [14], f, Eni w Eal f [24]
8	15.5	Digalloylglucose	483	313, 169	MS^2: 331, 327, **313**, 169; MS^3: 271, 211, 193, 169, 125	[14]	Egl b [14, 29], Egl f, Eni w Eco l [24]
9	16.0	Epicatechin	289	245, 205, 203, 179, 109		[32]	Egl b [29]
10	16.3	Quercetin-glucuronide	477	301	MS^2: **301**; MS^3: **179, 151**	[24]	Esp l [24]
11	17.7	Dihydroxy-isopropylchromone-hexoside	381	261, 233		[33]	RFE
12	18.9	Isorhamnetin-hexoside	477	315	MS^2: **315**; MS^3: **300**; MS^4: 271, 272, 244	[14]	Egl b [14]
13	19.4	Ellagic acid-rhamnoside	447	301	MS^2: **301**, 300; MS^3: 257, 229	[32]	RFE
14	20.0	Ellagic acid	301	229, 185, 173, 157, 146	-	Co	Esp b, f, l, w [14, 24]
15	21.2	Taxifolin	303	285, 177, 151, 125	-	[14]	Egl b [14, 25, 27, 34]
16	22.0	Quercetin-hexoside	463	301, 300	MS^2: **301,** 300; MS^3: 179, 151	[14]	Egl b [14], f, l, Eca Eru l Egu h [24] Ema l [17]

No.	Rt (min)	Compound	$[M-H]^-$ (*m/z*)	QqQ–MS^2 product ions (*m/z*)	IT–MS^n product ions (*m/z*)	Identified	Presence in *Eucalyptus* species[a]
17	22.8	Dihydroxy-(methylpropyl)isopropylchromone-hexoside	395	305, 275, 247	MS^2: **275**; MS^3: **247**, 191; MS^4: 204	[33]	RFE
18	23.3	Methyl-ellagic acid-pentoside	447	315	MS^2: **315**; MS^3: **300**	[14]	Egl f [24]
19	24.7	Myricetin-rhamnoside	463	317	-	[14]	Egl b [14]
20	25.1	Isorhamnetin-rhamnoside	461	315	MS^2: **315**; MS^3: **300**; MS^4: 272, 244	[14]	Egl b [14]
21	25.8	Aromadendrin-rhamnoside	433	287, 269, 259, 180, 179, 151	MS^2: **287,** 269; MS^3: **259**; MS^4: 241, 215, 125	[14]	Egl b [14, 27]
22	28.7	Mearnsetin	331	316	MS^2: **316**; MS^3: **287**,271; MS^4: 259	[14]	Egl b [14]
23	31.1	Mearnsetin-hexoside	493	331	MS^2: **331**; MS^3: **316**; MS^4: **287**, 271, 244; MS^5: 259	[14]	Egl b [14]
24	33.7	Eriodictyol	287	151, 135, 107	-	[14]	Egl b [14, 27, 34]
25	35.4	Quercetin	301	179, 165, 151, 121, 107	-	Co	Egl b [14, 27, 34], Ema l [17]
26	37.8	Isorhamnetin	315	300, 271	-	[14]	Egl b [14, 29, 34]
27	40.9	Naringenin	271	177, 151, 119, 107	-	Co	Egl b [14] Eca b [25], Eru l [26]

[a] Egl- *E. globulus,* E.ur- *E. urograndis,* Eca- *E. camaldulensis,* Eru- *E. rudis,* Ere- *E. regnans,* Eni-*E. nitens,* Eco- *E. consideniana,* Ecy- *E. cypellocarpa,* Egu- *E. gunnii,* Eal-*E. alba,* Esp- *Eucalyptus* species; Ema- *E. maidenii*, b-bark, l-leaves, f-fruits, w-wood, h-hook; RFE- Reported for the first time in *Eucalyptus* species; [b] Co = co-injection of the authentic standard; [c] *m/z* in bold was subjected to MS^n analysis

Most of the compounds identified in the presently studied species were previously found as constituents of *E. globulus* bark extracts [14], namely: quinic **1**, gallic **2**, protocatechuic **3**, chlorogenic **6** and ellagic acids **14**, methyl gallate **4**, catechin **5**, galloyl-bis-HHDP-glucose **7**, digalloylglucose **8**, isorhamnetin-hexoside **12**, taxifolin **15**, quercetin-hexoside **16**, methyl-ellagic acid-pentoside **18**, myricetin-rhamnoside **19**, isorhamnetin-rhamnoside **20**, aromadendrin-rhamnoside **21**, mearnsetin **22**, mearnsetin-hexoside **23**, eriodictyol **24**, quercetin **25**, isorhamnetin **26** and naringenin **27** (Figure 4.2). The relevant MS features of these compounds were previously discussed [14] (Chapter 3) and references therein.

Additionally, five other phenolic compounds (**9**, **10**, **11**, **13** and **17**) were identified in some of the studied barks (Figure 4.2). Epicatechin was identified as compound **9**, as confirmed by its $[M-H]^-$ at *m/z* 289, as well as to MS/MS product ions at *m/z* 245 $[M-H-CO_2]^-$, and at *m/z* 205 $[M-H-C_4H_4O_2]^-$, 203 $[M-H-C_4H_6O_2]^-$, due to cleavage of B ring and at *m/z* 179 $[M-H-C_6H_6O_2]^-$ and 109 $[B\ ring]^-$ [32]. Compound **10** was assigned to quercetin-glucuronide with $[M-H]^-$ at *m/z* 477 yielding a product ion at *m/z* 301, which corresponds to quercetin aglycone after the loss of a glucuronide moiety (-176 Da) [24]. The MS^n spectra and fragmentation pathway of this compound is shown in Figure 4.1.

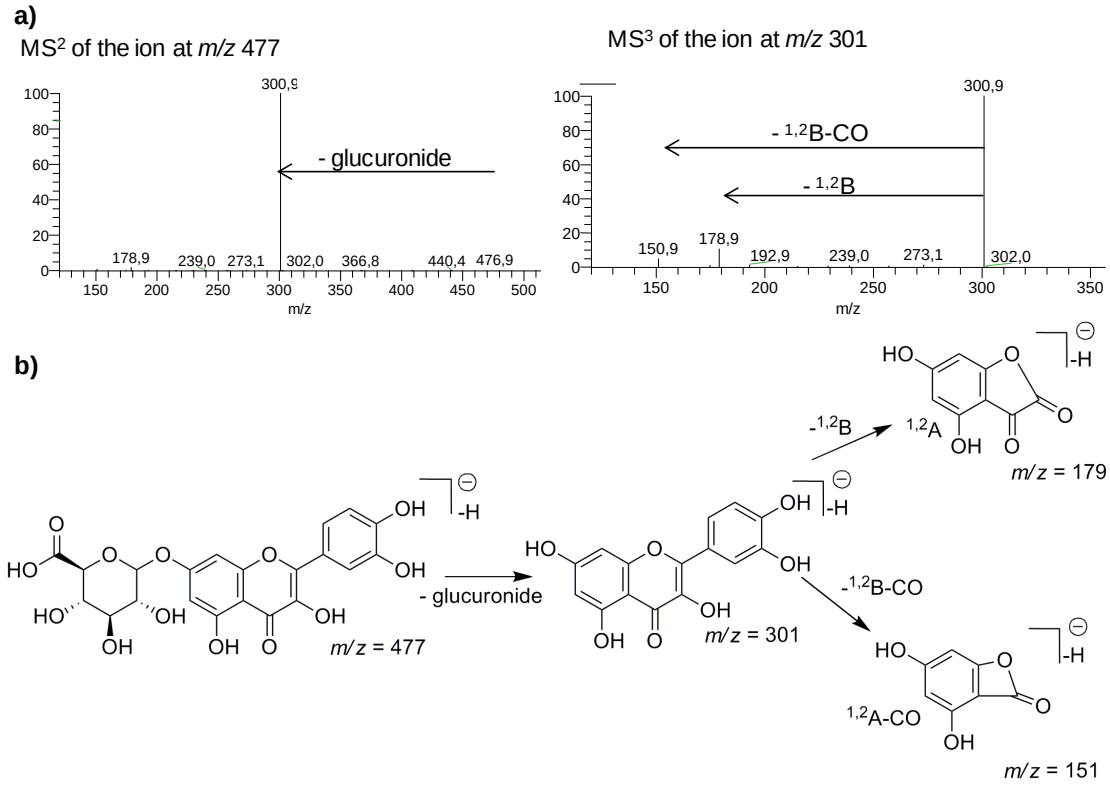


Figure 4.1 – a) MS^n spectra and b) fragmentation pathway of quercetin-glucuronide **10**

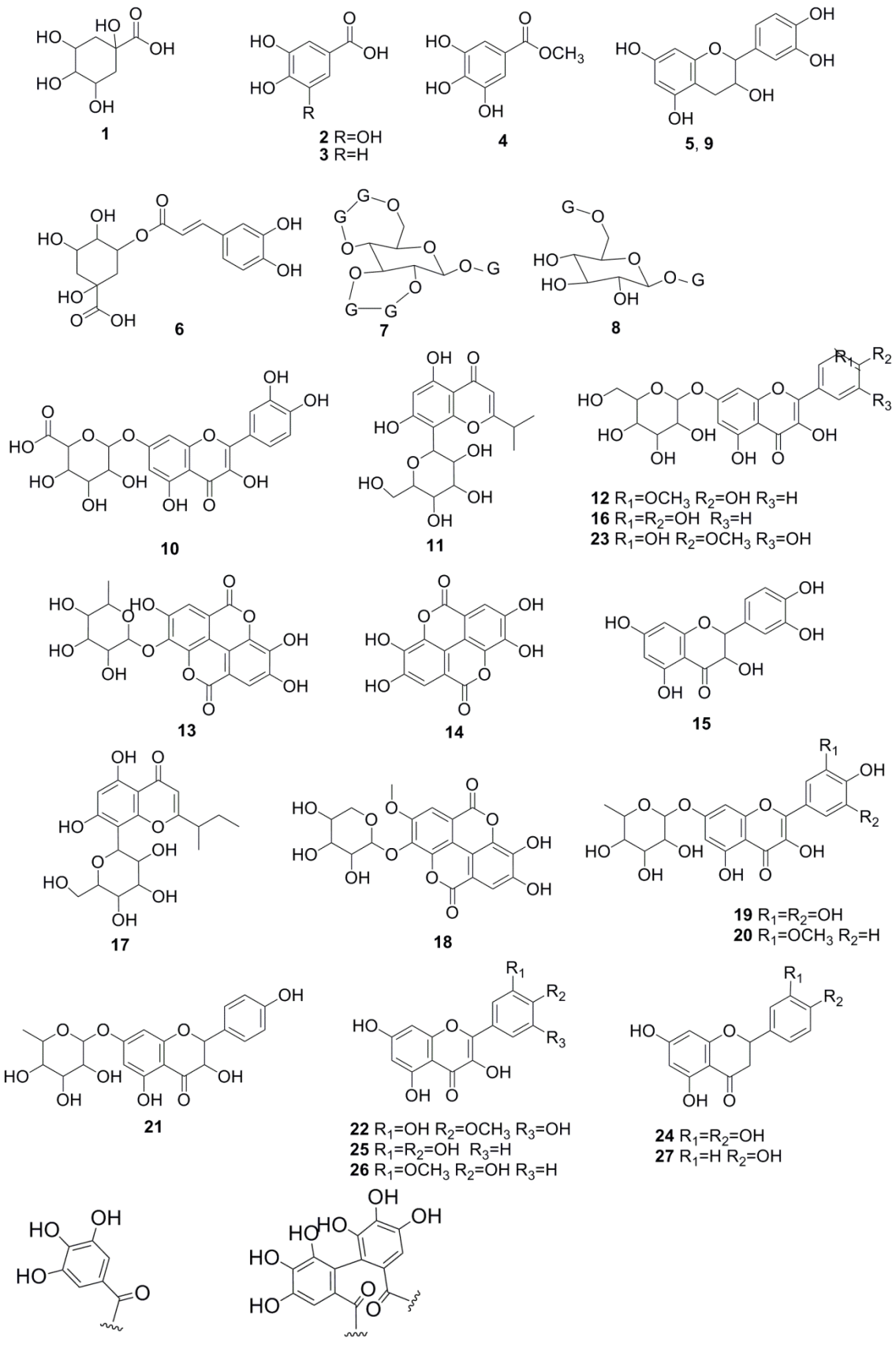


Figure 4.2 – Structures of the phenolic compounds identified in *E. grandis, E. urograndis* and *E. maidenii* bark extracts G:galloyl group; G-G: hexahydroxydiphenoyl (HHDP) group

Compound **11** was identified as dihydroxy-isopropylchromone-hexoside due to its $[M-H]^-$ at *m/z* 381 as well as to MS/MS product ions at *m/z* 261, resulting from the 0,2 cross-ring cleavage in the hexose $[M-H-120]^-$ and 233, resulting further from a loss of a carbonyl group $[M-H-120-28]^-$ [33]. Compound **13** was assigned to ellagic acid-rhamnoside, with $[M-H]^-$ at *m/z* 447 yielding the product ion at *m/z* 301 (-146 Da, -rhamnose). Furthermore, the identification of **13** was corroborated by the MS^3 product ions at *m/z* 257 and 229, showing the typical fragmentation profile of ellagic acid [32]. Finally, compound **17** was identified as dihydroxy-(methylpropyl)isopropylchromone-hexoside, based on its characteristic $[M-H]^-$ at *m/z* 395 and on the respective product ions at *m/z* 275 $[M-H-120]^-$ (cleavage in the 0,2 cross-ring of the hexose) and 247 $[M-H-120-28]^-$ (further loss of the carbonyl group) [33].

4.3.3 HPLC quantification of phenolic compounds

The phenolic composition of each extract quantified by HPLC is given in Table 4.4. *E. grandis* and *E. urograndis* have a similar composition, both in terms of the compounds identified, as well as their total abundance. Both extracts are constituted by quinic **1**, gallic **2**, protocatechuic **3** and ellagic acids **14**, methyl gallate **4**, catechin **5**, galloyl-bis-HHDP-glucose **7**, epicatechin **9**, quercetin-glucuronide **10**, ellagic acid-rhamnoside **13** and isorhamnetin-rhamnoside **20**. Additionally, digalloylglucose **8** and aromadendrin-rhamnoside **21** were detected in *E. grandis* and traces of naringenin **27** were detected in *E. urograndis*.

In *E. maidenii* bark we found most of the compounds reported above for *E. grandis* and *E. urograndis,* with the exception of digalloylglucose **8,** epicatechin **9** and quercetin-glucuronide **10**. In fact, this species shows a larger diversity on the phenolic components identified, namely chlorogenic acid **6**, dihydroxy-isopropylchromone-hexoside **11**, isorhamnetin-hexoside **12**, quercetin-hexoside **16**, dihydroxy-(methylpropyl)isopropylchromone-hexoside **17**, taxifolin **15**, methyl-ellagic acid-pentoside **18**, myricetin-rhamnoside **19**, mearnsetin **22**, mearnsetin-hexoside **23**, eriodictyol **24**, quercetin **25** and isorhamnetin **26**.

All the compounds are referenced for the first time as constituents of *E. grandis*, *E. urograndis* or *E. maidenii* bark, although gallic **2**, protocatechuic **3** and chlorogenic **6** acids have already been referenced as constituents of *E. urograndis* leaf litter [15]. Furthermore, ellagic acid-rhamnoside **13** and the chromones-hexosides **11** and **17** are referenced here for the first time as constituents of a morphological part of *Eucalyptus* species, despite they are commonly found in other natural sources [32, 33].

The quantification of the phenolic components of each extract by HPLC (Table 4.4) showed that both *E. grandis* and *E. urograndis* bark are rich in epicatechin **9** and quercetin-glucuronide **10** (quantified together, 68.20±0.67 and 118.86±0.71 mg g^{-1} of extract, respectively), followed by ellagic acid-rhamnoside **13** (47.32±0.42 mg g^{-1} of extract) and by ellagic acid **14** (25.43±0.39 mg g^{-1} of extract) in *E. grandis*, and by galloyl-bis-HHDP-glucose **7** (17.34±0.30 mg g^{-1} of extract) and gallic acid **2** (15.84±0.16 mg g^{-1} of extract) in *E. urograndis*.

E. maidenii was found to have lower amounts of the identified phenolic compounds, with catechin **5** (34.44±0.24 mg g^{-1} of extract), chlorogenic acid **6** (10.99±0.29 mg g^{-1} of extract) and methyl-ellagic acid-pentoside **18** (9.16±0.39 mg g^{-1} of extract) as the major components.

The total amounts of phenolic compounds identified by HPLC follows a trend similar to that of phenolic content by Folin-Ciocalteu method, with *E. grandis* with the higher value (213.13±2.57 mg g^{-1} of extract), followed closely by *E. urograndis* and by *E. maidenii.* However, the total amount of identified compounds per kg of bark is clearly higher in the case of *E. urograndis* (~28.9 g Kg^{-1}), due to its substantial higher extraction yield.

Finally, the total amounts of phenolic compounds identified in the barks of the three species analysed in this study are considerable superior to those recently reported for *E. globulus* bark (~10.9 g Kg^{-1} of bark) [14]. These results show the high potential of *E. grandis*, *E. urograndis* and *E. maidenii* barks as sources of valuable phenolic compounds.

Table 4.4 – HPLC quantification of phenolic components identified in *E. grandis*, *E. urograndis* and *E. maidenii* bark extracts

No.	Compound	λ (nm)	Phenolic content (mg g^{-1} of extract)		
			E. grandis	***E. urograndis***	***E. maidenii***
1	Quinic acid[a]	280	3.68±0.16	7.54±0.25	5.74±0.12
2	Gallic acid[a]	280	10.61±0.24	15.84±0.16	7.95±0.16
3	Protocatechuic acid[b]	280	1.83±0.06	1.27±0.06	1.17±0.04
4	Methyl gallate[a]	280	1.54±0.04	2.46±0.04	1.59±0.08
5	Catechin[c]	280	22.79±0.43	traces	34.44±0.24
6	Chlorogenic acid[d]	280	-	-	10.99±0.29
7	Galloyl-bis-HHDP-glucose[a]	280	15.81±0.19	17.34±0.30	8.44±0.37
8	Digalloylglucose[a]	280	14.75±0.11	-	-
9	Epicatechin[c]	280	68.20±0.67[h(9+10)]	118.86±0.71[h(9+10)]	-
10	Quercetin-glucuronide[c]	280[i]			-
11	Dihydroxy-isopropylchromone-hexoside[e]	280	-	-	2.70±0.09
12	Isorhamnetin-hexoside[e]	340	-	-	0.72±0.02
13	Ellagic acid-rhamnoside[f]	340	47.32±0.42	10.81±0.15	3.13±0.13
14	Ellagic acid[f]	340	25.43±0.39	14.99±0.21	8.72±0.18
15	Taxifolin[g]	280	-	-	4.91±0.15
16	Quercetin-hexoside[e]	340	-	-	2.18±0.10[h(16+17)]
17	Dihydroxy- (methylpropyl)isopropylchromone-hexoside	280	-	-	
18	Methyl-ellagic acid-pentoside[f]	340	-	-	9.16±0.39
19	Myricetin-rhamnoside[e]	340	-	-	5.27±0.17[h(19+20+21)]
20	Isorhamnetin-rhamnoside[e]	340	1.17±0.04[h(20+21)]	1.33±0.06	
21	Aromadendrin-rhamnoside[e]	340[i]		-	

No.	Compound	λ (nm)	Phenolic content (mg g^{-1} of extract)		
			E. grandis	*E. urograndis*	*E. maidenii*
22	Mearnsetin[e]	340	-	-	0.15±0.01
23	Mearnsetin-hexoside[e]	340	-	-	0.25±0.01
24	Eriodictyol[g]	280	-	-	0.93±0.04
25	Quercetin[e]	340	-	-	0.16±0.01
26	Isorhamnetin[e]	340	-	-	0.95±0.04
27	Naringenin[g]	280	-	traces	0.87±0.01
Total (mg g^{-1} of extract)			213.13±2.57	190.44±0.31	110.43±0.23
Total (mg kg^{-1} of bark)			22463.96±271.37	28908.54±47.04	14609.40±30.77

All values are expressed as mean±standard deviation (n=3)
Calibrations curved used: [a] Gallic acid, [b] Protocatechuic acid, [c] Catechin, [d] Chlorogenic acid, [e] Quercetin, [f] Ellagic acid, [g] Naringenin;
[h] Sum of the phenolic content by partial overlapping of the identified peaks; i Due to the overlapped peaks it was chosen the wavelength where the peak area was higher.

4.3.4 Antioxidant activity

Table 4.5 presents the results of the antioxidant activity obtained for the studied extracts, expressed in terms of the amount of extract needed to decrease the DPPH concentration by 50% (IC_{50}), as well as in terms of the ascorbic acid equivalents (AAE) on a bark basis (mg AAE g^{-1} of bark). The IC_{50} values for ascorbic acid and for BHT were also obtained and reported in Table 4.5 for comparative purposes.

Table 4.5 – Antioxidant activity of *E. grandis*, *E. urograndis* and *E. maidenii* bark extracts by DPPH radical scavenging

	IC_{50} (µg mL^{-1})	**values in ascorbic acid equivalents (mg AAE[a] g^{-1} of bark)**
Ascorbic acid	2.15±0.07	-
BHT	18.22±0.21	-
E. grandis	6.26±0.19	36.73±0.89
E. urograndis	6.14±0.21	53.18±1.80
E. maidenii	8.24±0.26	34.54±1.12

All values are expressed as mean±standard deviation (n=3); [a] AAE: ascorbic acid equivalents

These extracts have revealed an antioxidant activity considerably higher than that reported for BHT and lower of that measured for ascorbic acid. Furthermore, the antioxidant activities showed similar trends to those previously mentioned to phenolic content: *E. grandis* and *E. urograndis* show a similar antioxidant scavenging and higher than *E. maidenii* bark extract, in line with the amounts of phenolic components detected by HPLC-MS and with the reported total phenolic content values.

Finally, *E. urograndis* bark extract reached the higher antioxidant potential, when the antioxidant capacity is expressed as AAE on a bark basis, due to its higher extraction yield (Table 4.2). Nevertheless, all the extracts present excellent values of AAE per Kg of bark, significantly higher than that reported for *E. globulus* bark extracts [23] or for the barks of other species as *Q. suber* extracts [21].

4.4 Conclusions

To our knowledge, this is the first study concerning the analysis of the phenolic composition of *E. grandis*, *E. urograndis* and *E. maidenii* barks by HPLC–MS/MS and MS^n. Thirteen, twelve and twenty four phenolic compounds were identified in *E. grandis*, *E. urograndis* and *E. maidenii* bark extracts, respectively. Furthermore, ellagic

acid-rhamnoside **13,** dihydroxy-isopropylchromone-hexoside **11** and dihydroxy-(methylpropyl)isopropylchromone-hexoside **17** are referenced for the first time as constituents of *Eucalyptus* species. Epicatechin **9** and quercetin-glucuronide **10** are the major phenolic compounds in *E. grandis* and *E. urograndis* bark, followed by ellagic acid-rhamnoside **13** and ellagic acid **14** in *E. grandis* and by galloyl-bis-HHDP-glucose **7** and gallic acid **2** in *E. urograndis*. *E. maidenii* bark is rich in catechin **5,** chlorogenic acid **6** and methyl-ellagic acid-pentoside **18**. The phenolic composition of the three extracts shows a positive correlation with their antioxidant activities, determined by DPPH radical scavenging, being the activities values between those of two commercial antioxidants, namely, ascorbic acid and BHT. Additionally, the antioxidant potentials become significantly higher when expressed *per* mass unit. These results are promising, confirming, together with the phenolic composition verified, the vast potential of these species as source of biologically active phenolic compounds.

References

[1] Ugalde, L.; Pérez, O. Mean annual volume increment of selected industrial forest plantation species. www.fao.org/forestry (August 2011)

[2] Trabado, G. I.; Wilstermann, D. Cultivated Eucalyptus Forests Global Map 2008. http://www.git-forestry.com (August 2011)

[3] Quilhó, T.; Miranda, I.; Pereira, H., Within-tree variation in wood fibre biometry and basic density of the urograndis eucalypt hybrid (*Eucalyptus grandis* x *E. urophylla*). *IAWA J.* **2006,** *27*, 243-254.

[4] ABRAF. ABRAF Statistical yearbook - base year 2010. http://www.abraflor.org.br (September 19, 2012)

[5] Kibblewhite, R. P.; Johnson, B. I.; Shelbourne, C. J. A. In *Kraft pulp qualities of Eucalyptus nitens, E. globulus, and E. maidenii, at ages 8 and 11 years*, 55th Appita Annual Conference Proceedings, Hobart, Australia, 2001; Hobart, Australia, 2001.

[6] Fernando, S.; Adhikari, S.; Chandrapal, C.; Murali, N., Biorefineries: Current status, challenges, and future direction. *Energ. Fuel* **2006,** *20*, 1727-1737.

[7] Kamm, B.; Kamm, M.; Schimdt, M.; Hirth, T.; Schulze, M., Lignocellulose-based chemical products and product family trees. In *Biorefineries- Industrial Processes and Products: Status Quo and Future Directions*, Kamm, B.; Gruber, P.; Kamm, M., Eds. Wiley VCH: Weinheim, Germany, 2006; Vol. 2, pp 97-150.

[8] Ragauskas, A. J.; Williams, C. K.; Davison, B. H.; Britovsek, G.; Cairney, J.; Eckert, C. A.; Frederick, W. J.; Hallett, J. P.; Leak, D. J.; Liotta, C. L.; Mielenz, J. R.; Murphy, R.; Templer, R.; Tschaplinski, T., The Path Forward for Biofuels and Biomaterials. *Science* **2006,** *311*, 484-489.

[9] Pietarinen, S.; Willför, S.; Ahotupa, M.; Hemming, J.; Holmbom, B., Knotwood and bark extracts: strong antioxidants from waste materials. *J. Wood Sci.* **2006,** *52*, 436-444.

[10] Hamunen, A. Process for the purification of *β*-sitosterol isolated from the unsaponifiables in crude soap from the sulphate cellulose process. 1983.

[11] Freire, C. S. R.; Silvestre, A. J. D.; Neto, C. P.; Cavaleiro, J. A. S., Lipophilic extractives of the inner and outer barks of *Eucalyptus globulus*. *Holzforschung* **2002,** *56*, 372-379.

[12] Domingues, R. M. A.; Sousa, G. D. A.; Freire, C. S. R.; Silvestre, A. J. D.; Neto, C. P., *Eucalyptus globulus* biomass residues from pulping industry as a source of high value triterpenic compounds. *Ind. Crop Prod.* **2010,** *31*, 65-70.

[13] Domingues, R. M. A.; Sousa, G. D. A.; Silva, C. M.; Freire, C. S. R.; Silvestre, A. J. D.; Neto, C. P., High value triterpenic compounds from the outer barks of several Eucalyptus species cultivated in Brazil and in Portugal. *Ind. Crop. Prod.* **2011,** *33*, 158-164.

[14] Santos, S. A. O.; Freire, C. S. R.; Domingues, M. R. M.; Silvestre, A. J. D.; Neto, C. P., Characterization of phenolic components in polar extracts of *Eucalyptus globulus* Labill. bark by high-performance liquid chromatography-mass spectrometry. *J. Agric. Food Chem.* **2011,** *59*, 9386-9393.

[15] Chapuis-Lard, L.; Contour-Ansel, D.; Bernhard-Reversat, F., High-performance liquid chromatography of water-soluble phenolic compounds in leaf litter of three Eucalyptus hybrids (Congo). *Plant Sci.* **2002,** *163*, 217-222.

[16] Kulkarni, A.; Suzuki, S.; Etoh, H., Antioxidant compounds from *Eucalyptus grandis* biomass by subcritical liquid water extraction. *J. Wood Sci.* **2008,** *54*, 153-157.

[17] Manguro, L. O. A.; Mukonyi, K. W.; Githiomi, J. K., A New Flavonol Glycoside from Eucalyptus-Globulous Subsp Maidenii. *Nat. Prod. Lett.* **1995,** *7*, 163-167.

[18] Hassan Khan, M. T.; Ather, A.; El-Gewely, M. R., Potentials of phenolic molecules of natural origin and their derivatives as anti-HIV agents. In *Biotechnology Annual Review*, El-Gewely, M. R., Ed. Elsevier: 2007; Vol. Volume 13, pp 223-264.

[19] Carrol, K. K.; Kurowaska, E. M.; Guthrie, N. Use of citrus limonoids and flavonoids as well as tocotrienols for the treatment of cancer. 1999.

[20] Singleton, V. L.; Rossi, J. A., Jr., Colorimetry of Total Phenolics with Phosphomolybdic-Phosphotungstic Acid Reagents. *Am. J. Enol. Vitic.* **1965,** *16*, 144-158.

[21] Santos, S. A. O.; Pinto, P.; Silvestre, A. J. D.; Neto, C. P., Chemical composition and antioxidant activity of phenolic extracts of cork from *Quercus suber* L. *Ind. Crop. Prod.* **2010,** *31*, 521-526.

[22] Sharma, O. P.; Bhat, T. K., DPPH antioxidant assay revisited. *Food Chem.* **2009,** *113*, 1202-1205.

[23] Vazquez, G.; Gonzalez-Alvarez, J.; Santos, J.; Freire, M. S.; Antorrena, G., Evaluation of potential applications for chestnut (*Castanea sativa*) shell and eucalyptus (*Eucalyptus globulus*) bark extracts. *Ind. Crop. Prod.* **2009,** *29*, 364-370.

[24] Boulekbache-Makhlouf, L.; Meudec, E.; Chibane, M.; Mazauric, J. P.; Slimani, S.; Henry, M.; Cheynier, V.; Madani, K., Analysis by High-Performance Liquid Chromatography Diode Array Detection Mass Spectrometry of Phenolic Compounds in Fruit of Eucalyptus globulus Cultivated in Algeria. *J. Agric. Food Chem.* **2010,** *58*, 12615-12624.

[25] Conde, E.; Cadahía, E.; Diez-Barra, R.; García-Vallejo, M., Polyphenolic composition of bark extracts from *Eucalyptus camaldulensis*, *E. globulus* and *E. rudis*. *Holz als Roh- und Werkstoff* **1996,** *54*, 175-181.

[26] Conde, E.; Cadahía, E.; García-Vallejo, M. C., Low molecular weight polyphenols in leaves of *Eucalyptus camaldulensis*, *E. globulus* and *E. rudis*. *Phytochem. Analysis* **1997,** *8*, 186-193.

[27] Yun, B.-S.; Lee, I.-K.; Kim, J.-P.; Chung, S.-H.; Shim, G.-S.; Yoo, I.-D., Lipid peroxidation inhibitory activity of some constituents isolated from the stem bark of *Eucalyptus globulus*. *Arch. Pharm. Res.* **2000,** *23*, 147-150.

[28] Hou, A. J.; Liu, Y. Z.; Yang, H.; Lin, Z. W.; Sun, H. D., Hydrolyzable tannins and related polyphenols from *Eucalyptus globulus*. *J. Asian Nat. Prod. Res.* **2000,** *2*, 205-212.

[29] Vázquez, G.; Santos, J.; Freire, M.; Antorrena, G.; González-Álvarez, J., Extraction of antioxidants from eucalyptus *Eucalyptus globulus* bark. *Wood Sci. Technol.* **2011**, 1-15.

[30] Yazaki, Y.; Hillis, W. E., Polyphenols of *Eucalyptus globulus*, *E. Regnans* and *E. Deglupta*. *Phytochemistry* **1976,** *15*, 1180-1182.

[31] Ito, H.; Koreishi, M.; Tokuda, H.; Nishino, H.; Yoshida, T., Cypellocarpins A-C, phenol glycosides esterified with oleuropeic acid, from *Eucalyptus cypellocarpa*. *J. Nat. Prod.* **2000,** *63*, 1253-1257.

[32] Sandhu, A. K.; Gu, L. W., Antioxidant Capacity, Phenolic Content, and Profiling of Phenolic Compounds in the Seeds, Skin, and Pulp of *Vitis rotundifolia* (Muscadine Grapes) As Determined by HPLC-DAD-ESI-MS^n. *J. Agric. Food Chem.* **2010,** *58*, 4681-4692.

[33] Gao, W.-N.; Luo, J.-G.; Kong, L.-Y., Quality evaluation of *Hpericum japomicum* by using high-performance liquid chromatography coupled with photodiode array detector and electrospray ionization tandem mass spectrometry. *Biomed. Chromatogr.* **2009,** *23*, 1022-1030.

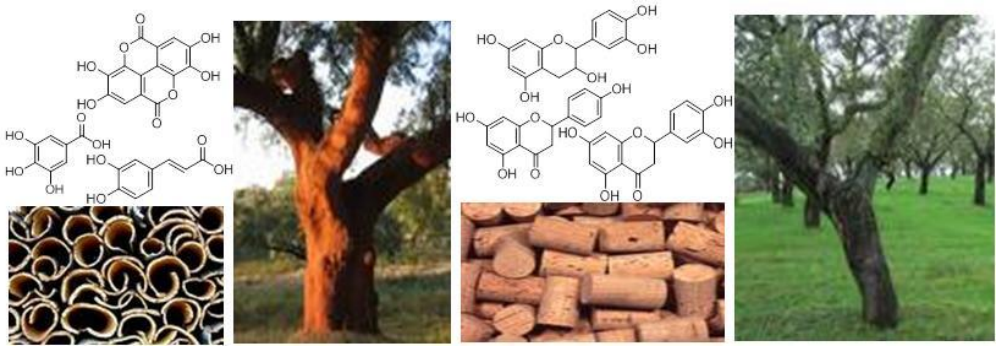
[34] Conde, E.; Cadahía, E.; García-Vallejo, M.; Tomás-Barberán, F., Low molecular weight polyphenols in wood and bark of *Eucalyptus globulus*. *Wood Fiber Sci.* **1995,** *27*, 379-383.

Chapter 5

Characterisation of phenolic fraction from *Quercus suber* L. cork and cork by-products

Part A

Characterisation of phenolic fraction of *Quercus suber* L. cork



Adapted from:

Santos, S.A.O. Pinto, P.C.R.O., Silvestre, A.J.D., Neto, C.P., Chemical composition and antioxidant activity of phenolic extracts of cork from *Quercus suber* L., *Industrial Crops and Products*, **2010**, 31, 521-526

Abstract

The phenolic fraction of cork from *Quercus suber* L. was obtained following two distinct fractionation schemes, namely, methanol/water extraction followed by ethyl ether fractionation, and sequential extraction with methanol and water. The extracts were studied in terms of total phenolic compounds content, using Folin-Ciocalteu method, detailed chemical analysis by HPLC–MS, and antioxidant activity. The first method underestimates both total extractives, total phenolic content as well as the amounts of identified compounds. The HPLC–MS, revealed that, apart from smaller components, all the extracts displayed the same qualitative composition; 15 phenolic components were identified, with ellagic acid, followed by gallic and protocatechuic acids as the most abundant compounds. Additionally, some compounds identified were reported for the first time as cork components, namely salicylic acid, eriodictyol, naringenin, quinic acid and hydroxyphenyllactic acid. The antioxidant activity of the extracts, evaluated using the 2,2-diphenyl-2-picrylhydrazyl hydrate (DPPH) radical scavenging assay, showed to be considerably higher than that of BHT, and in the range of ascorbic acid. The antioxidant potential per mass unit of the three extracts is in the same range, but the high extraction yields obtained by water extraction open good perspectives for the exploitation of this extract in nutraceutical applications.

5.1 Introduction

The up-grading of the by-products of the forestry industry constitutes an important challenge on the development of a sustainable economy and of environmentally friendly industrial processes. These by-products are seen, in recent years, as promising sources of renewable chemicals, materials and fuels and as a response to the inevitable depletion of fossil resources within the emerging “biorefinery” concept [1, 2].

Cork is the outer bark of *Quercus suber*, a common species in the Mediterranean region. Because of its peculiar properties [3-5], such as high elasticity and low permeability, cork has a large variety of applications, among which the production of stoppers for wine and other alcoholic beverages is by far the most important, followed by its applications in thermal and/or acoustic insulation materials. Portugal produces about 157 000 ton of cork/year, which represents about 53% of the world production [6]. This industry generates substantial amounts of a residue, namely industrial cork powder, which represents in Portugal, about 34 000 ton/year, more than 20% of the total cork production. This by-product, that is generally not suitable for current industrial uses, is currently mostly burned to produce energy [7]. The full exploitation of this resource and specially the detailed study of its chemical composition is a key step towards the recovery of this by-product.

Cork is mainly composed of lignin (25%, w/w), polysaccharides (20%, w/w), suberin (40%, w/w), extractives (15%, w/w), and inorganics (1%, w/w) [8, 9]. Suberin, due to its abundance and unique composition, is the most promising component for the development of new chemicals and materials from cork by-products [3, 5, 10, 11]. However, in an integrated biorefinery perspective, all cork components, extractives included, should be considered. Cork extractives are mainly composed of aliphatic, phenolic and triterpenic components. The detailed chemical composition of the lipophilic extractives of cork and cork by-products has been recently investigated by Sousa *et al.* [12], demonstrating that this fraction could be an interesting source of bioactive triterpenic compounds.

However, the information available on the phenolic fraction of cork is scarce, despite the fact that this group of components can also be easily extracted from those residues. The total contents of polymeric polyphenols from cork (lignins and tannins), have been reported [8, 13], as well as some low molecular weight phenolic constituents

of suberin [14, 15]. Conde *et al.* [16, 17] have reported the presence of several phenolic acids, namely ellagic (also reported by Sousa *et al.* [12]), gallic, caffeic and protocatechuic acids, together with vanillin, protocatechuic aldehyde, coniferaldehyde and sinapaldehyde in extracts of cork from *Q. suber* L. The presence of ellagitannins, namely roburin, grandinin, and castalagin in extracts of cork [18] has also been reported.

The interest on natural phenolic compounds relies on the wide variety of relevant properties shown by this family, namely, among others, their antioxidant, anti-inflammatory, radical scavenger and antimicrobial properties [19-21]. The interest in natural phenolic compounds for nutraceutical and cosmetic applications has increased considerably in recent years because of the mentioned properties but also because they do not show adverse effects as it is frequently the case of their synthetic counterparts [22].

In addition, the number of studies on the composition of the phenolic fraction of cork extractives is limited; additionally, none of those studies accessed the antioxidant activity of the extracts. In this perspective, and within a wider project aiming at developing new strategies for the up-grading of cork by-products [9, 12], in the present work we report the quantification of total phenolic compounds content and detailed characterisation of the cork phenolic fraction by HPLC–MS, obtained by methanol and water extraction. For comparative purposes, the extracts and fractions obtained following a previously published procedure [16] were analysed. The extracts were evaluated in terms of their antioxidant properties, using the 2,2-diphenyl-2-picrylhydrazyl hydrate (DPPH) radical scavenging assay [23].

5.2 Materials and methods

5.2.1 Chemicals

Gallic acid, Folin-Ciocalteu's phenol reagent, 2,2-diphenyl-2-picrylhydrazyl hydrate (DPPH) and 3,5-di-*tert*-4-butylhydroxytoluene (BHT) were supplied by Sigma Chemicals Co. Diethyl ether, and the HPLC-grade methanol, water and acetonitrile, were obtained from Fisher Scientific Chemicals. Formic acid, methanol, ascorbic acid and ellagic acid were purchased from Fluka Chemie. Vanillin, vanillic acid and ferulic acid were supplied by Aldrich Chemicals Co. The other phenolic compounds used, were isolated in our department from other plant sources.

All other chemicals and solvents were available in our laboratories and used without any further purification.

5.2.2 Raw material

Q. suber L. natural (WNC) cork planks ("amadia" grade) were sampled from the south of Portugal (Herdade da Moinhola, Amorim Florestal). An average sample composed of fragments of several planks from different trees was milled in a Retsch cross beater mill SK1 (Haan, Germany), and the granulometric fraction of 40–60 mesh was used for analyses.

5.2.3 Phenolic compounds extraction

About 20 g of the cork powder sample was submitted to a soxhlet extraction with dichloromethane for 6 h, to remove the lipophilic components. Then, the solid cork residue was divided into two fractions (I and II), which followed distinct extraction pathways. Fraction I was suspended in a methanol–water mixture, 80/20 (v/v), at room temperature for 24 h under constant stirring [16]. The suspension was then filtered and MeOH removed by low-pressure evaporation. The methanol free aqueous solution was then extracted three times with diethyl ether, and the solvent was then removed in the rotary evaporator yielding MeOH/H_2O extract.

Fraction II was submitted to a methanol extraction for 6 h, and then by a reflux with water for 6 h. The solvents were subsequently removed from the liquid extracts by low-pressure evaporation and freeze drying yielding MeOH and H_2O extracts, respectively.

5.2.4 HPLC–MS analysis

Liquid chromatography of the extracts was carried out on a HPLC system HP 1050 equipped with a Rheodyne injector with a10 µL loop, a quaternary pumping system and a UV detector. The wavelengths used to detect the phenolic compounds were 280 and 340 nm. The column used was a Discovery® C-18 (15 cm × 2.1 mm × 5 µm) supplied by Supelco. The elution was performed with water (A) and acetonitrile (B) both containing 0.1% of HCOOH; the gradient profile was as follows: 0 min, 10% B; 80 min, 100% B and then held for 30 min before returning to the initial conditions. The flow rate was 0.2 mL min^{-1}. Mass spectrometry analysis was performed using a Micromass spectrometer (Manchester, UK) equipped with an electrospray source and a triple quadrupole analyser. The cone voltage was between -30 and -50 V, and the capillary voltage ranged from -2.6 to -2.9 kV. The source temperature was 143 ºC and the desolvation temperature was 350 ^{o}C. MS/MS spectra in the negative mode were

obtained using argon as collision gas with the collision energy set between 10 and 45 V.

5.2.5 HPLC-UV quantification

Gallic and ellagic acids were used as reference compounds for quantitative analysis, at 280 and 340 nm, respectively and with concentrations ranging between 0.05 and 1.5 mg mL^{-1}. The peaks areas were obtained with the UV detector monitored at 280 nm to gallic acid and at 340 nm to ellagic acid. The calibration curves obtained were: $C_{280\ nm}$ (mg gallic acid equivalents mL^{-1}) = 1.80 x 10^{-6}.area (R^2 = 0.992) and $C_{340\ nm}$ (mg ellagic acid equivalents mL^{-1}) = 1.88 x 10^{-6}.area (R^2 = 0.996). Phenolic compounds concentrations were calculated in triplicate and the mean value calculated in each case.

5.2.6 Total phenolic content

The total phenolic content of the extracts was determined by the Folin-Ciocalteu method [24], as described before (Chapter 3). 2.5 mL of Folin-Ciocalteu reagent, previously diluted with water (1:10, v/v), and 2 mL of aqueous sodium carbonate (75 g L^{-1}) were added to accurately weighed aliquots of the extracts dissolved in 0.5 mL of water for the H_2O extract, and in methanol for the others, corresponding to concentrations ranging between 40 and 310 µg of extract mL^{-1}. Each mixture was kept for 5 min at 50 ºC and, after cooling, the absorbance was measured at 760 nm, using a UV/Vis V-530 spectrophotometer (Jasco, Tokyo, Japan). The total phenolic content was calculated as gallic acid equivalent from the calibration curve of gallic acid standard solutions (1.5–60.0 µg mL^{-1}) and expressed as g gallic acid equivalent (GAE) g^{-1} of dry extract and as g GAE kg^{-1} of dry cork. The analyses were carried out in triplicate and the average value was calculated in each case.

5.2.7 Antioxidant activity

The antioxidant activity of the extracts was determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging [23] and as described in Chapter 3. The concentration of extracts ranged between 0.3 and 90 µg mL^{-1} for MeOH/H_2O and MeOH extracts and between 2.5 and 10 µg mL-1 for H_2O extract. Ascorbic acid and 3,5-di-*tert*-4-butylhydroxytoluene (BHT) were used as reference compounds. Triplicate measurements were carried out. The antioxidant activity was expressed as IC_{50} values (µg mL^{-1}) and also expressed in g of ascorbic acid equivalents kg^{-1} of dry cork (g AAE kg^{-1} of dry cork).

5.3 Results and discussion

5.3.1 Extraction yields and total phenolic content

The extraction yields of the studied cork extracts and the corresponding total phenolic contents are shown in Table 5.1. These extraction yields were obtained after removal of the dichloromethane soluble fraction, which accounted for 3.6% of cork weight, in agreement with previously published data [12]. The yields found are within the typical values found for *amadia* grade cork [8, 12]. The total phenolic contents, determined by the Folin-Ciocalteu method for the three extracts are in range of 0.20–0.35 g of gallic acid equivalents g^{-1} of extract, however, when expressed in g GAE kg^{-1} cork, the total phenolic content is significantly higher in the water extract, followed by the methanol extract, as a consequence of the corresponding increasing extraction yields. The total phenolic content for cork extracts ranged from 2.4 to 10.6 g kg^{-1} of dry cork extracts, which are within the range of those obtained for a large variety of plant materials [25, 26]. Likewise, the phenolic content for MeOH/H_2O extract presents a value of 2.4 g GAE kg^{-1} of dry cork, which is coincident with those previously reported for diethyl ether extracts from *Q. suber* cork [16-18]. However, when considered together, MeOH and H_2O extracts account for 16.5 g GAE kg^{-1} of dry cork, a value considerably higher than that of MeOH/H_2O extract, obtained through the fractionation method proposed by Conde *et al.* [16]. These results show that a considerable fraction of phenolic components are not extracted due to mild conditions of MeOH:H_2O extraction and also to the subsequent ethyl ether extraction, both limiting the yield of MeOH/H_2O extract.

Table 5.1 – Extraction yields and total phenolic contents of cork extracts from *Q. suber* L.

Extract	Extraction yield (%)	Total phenolic content	
		(g GAE g^{-1} of extract)	(g GAE kg^{-1} of dry cork)
MeOH/H_2O	1.2	0.20 ± 0.04	2.4 ± 0.5
MeOH	1.7	0.35 ± 0.01	5.9 ± 0.1
H_2O	3.7	0.29 ± 0.02	10.6 ± 0.6

Finally, the high content of phenolic components in the water extract shows that it is possible to use water instead of more harmful systems to isolate this fraction, which could be particularly interesting when nutraceutical applications are searched for these extracts.

5.3.2 Identification of phenolic compounds

The HPLC–MS identification of phenolic compounds was carried out by comparing peak retention times and fragmentation profiles with reference compounds run under the same experimental conditions and/or with published data. The phenolic compounds identified in the studied extracts, as well as their quantification, retention time, molecular ion $[M-H]^-$ values and the corresponding MS/MS fragmentation peaks are reported in Table 5.2.

Compounds were identified by comparison of their retention times and fragmentation patterns with those of standard compounds or by comparison with published data (see Table 5.2). Some of these compounds were previously reported in the literature [17, 27] as cork components, namely gallic **2**, protocatechuic **3** and caffeic **7,** vanillic **9**, *p*-coumaric **10**, ferulic **11** and *p*-hydroxybenzoic **5** acids and esculetin **6** and vanillin **8**. In addition to the known compounds, the ESI-MS analysis also allowed to identify, for the first time in cork extracts, several components, namely, salicylic acid **13**, eriodictyol **14** and naringenin **15** in MeOH/H_2O extract, quinic acid **1** in MeOH extract and *p*-hydroxyphenyllactic **4** acid in H_2O extract.

Table 5.2 – HPLC–MS evaluation of the extracts of cork from *Q. suber* L. expressed as mg kg^{-1} of dry cork (compounds in bold were identified for the first time in this species).

Nº	Rt (min)	Compound	λ (nm)	Phenolic content (mg kg^{-1} dry cork)			MS Data		Identified
				MeOH/H_2O	MeOH	H_2O	$[M-H]^-$	ESI-MS/MS fragments	
1	2.80	**Quinic acid**	280	-	TR	-	191	173, 127, 111, 93, 85	[28]
2	3.86	Gallic acid	280	30.6	48.1	241.6	169	125	Co
3	6.27	Protocatechuic acid	280	17.5	59.0	118.3	153	109	Co
4	6.71	***p*-Hydroxyphenyllactic acid**	280	–	–	TR	181	137, 113, 109	
5	9.55	*p*-Hydroxybenzoic acid	280	–	–	1.0	137	93	Co
6	11.20	Esculetin	280	4.9	106.7	–	177	133, 105	[29]
7	11.56	Caffeic acid	280	57.6	-	12.9	179	135	Co
8	13.97	Vanillin	280	14.3	TR	–	151	136	Co
9	14.96	Vanillic acid	280	TR	TR	–	167	152, 108	[30]
10	15.13	*p*-Coumaric acid	280	TR	–	–	163	119, 95	Co
11	16.25	Ferulic acid	340	TR	–	–	193	178, 149, 134, 117	Co
12	16.42	Ellagic acid	340	2031.5	1576.9	526.5	301	284, 245, 229, 201, 185, 173, 157, 145	Co
13	20.32	**Salicylic acid**	280	32.7	–	–	137	93	Co
14	24.72	**Eriodictyol**	280	27.4	–	–	287	151, 135,	[31]
15	28.41	**Naringenin**	280	2.6	–	–	271	177, 151, 119, 107	Co
		Total (mg kg^{-1} dry cork)		**2219.1**	**1790.7**	**900.3**			
		Total (mg g^{-1} extract)		**184.9**	**105.3**	**24.3**			

TR, identified compound but not possible to quantify by overlapping of peaks in HPLC chromatogram.
Co, identified by co-injection and ESI fragmentation of a reference sample.

5.3.2.1 Phenolic acids, aldehydes and derivatives

Quinic acid **1** (Figure 5.1) shows a fragmentation in accordance with the literature [28], with a $[M-H]^-$ ion at *m/z* 191 and product ions at *m/z* 173, 127, 111, 93 and 85. 181. Quinic acid **1** is a derivative of chlorogenic acid, which can be found in many plant species [28], including teas [32], but it has never been reported in cork extracts. Quinic acid is used as precursor of the synthesis of compounds with pharmaceutical applications and, interestingly, in the treatment of some influenza strains [33].

Peaks **2**, **3**, **5, 8, 12** and **13** were assigned to gallic, protocatechuic and *p*-hydroxybenzoic acids, vanillin and ellagic and salicylic acids (Figure 5.1), respectively, with their retention time, $[M-H]^-$ ions and corresponding product ions in accordance with the respective standards.

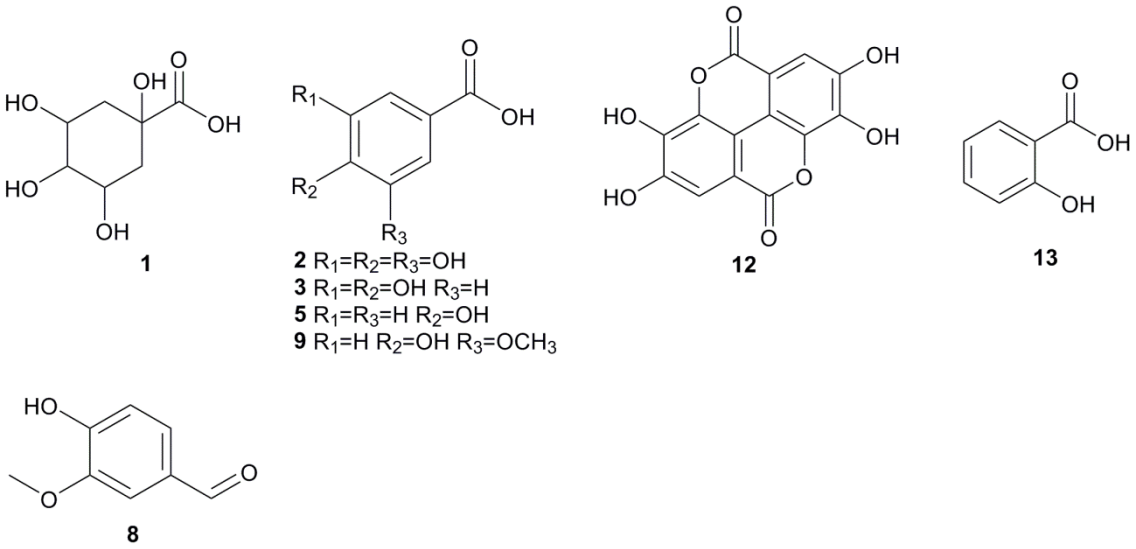


Figure 5.1 – Structures of phenolic acids, aldehydes and derivatives identified in *Q. suber* cork

Compound **9** was identified as vanillic acid (Figure 5.1) based on its $[M-H]^-$ ion at *m/z* 167 and the respective MS/MS product ions at *m/z* 152 and 108, corresponding to the loss of methyl group followed by the loss of carboxylic group (Figure 5.2). This identification was corroborated with the literature [30].

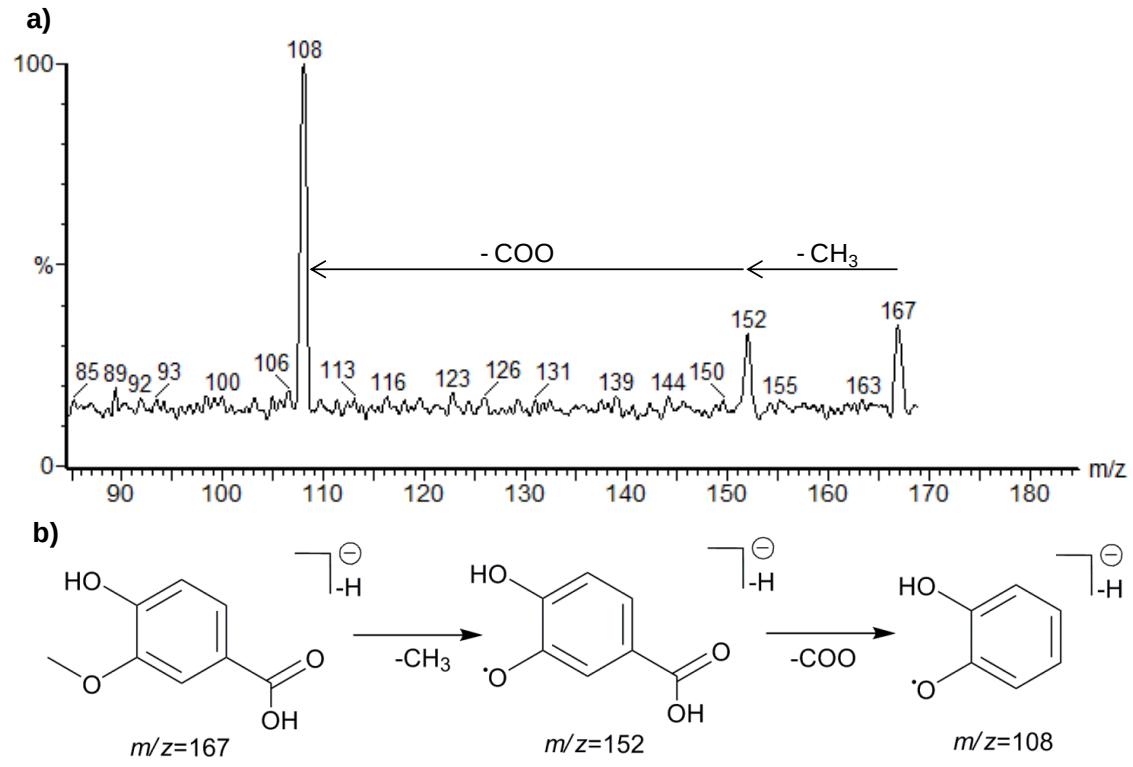


Figure 5.2 – a) MS/MS spectrum and b) fragmentation pathway of vanillic acid **9**

5.3.2.2 Cinnamic acids

Compounds **7**, **10** and **11** were identified as caffeic, *p*-coumaric and ferulic acids (Figure 5.3), respectively, by comparing their retention times and fragmentation pathways observed in the MS/MS spectra with those of the corresponding reference compounds.

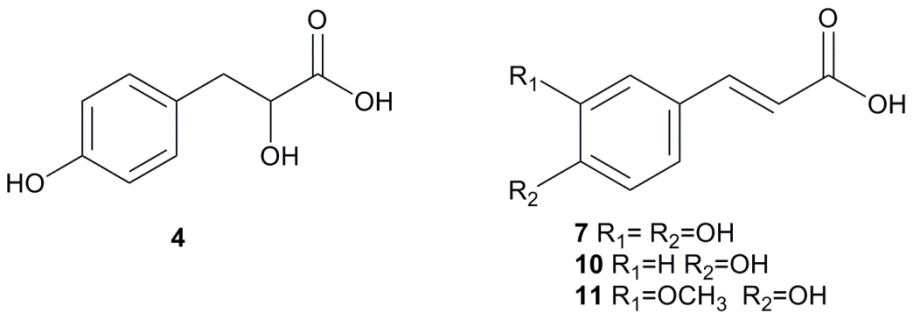


Figure 5.3 – Structures of cinnamic acids identified in *Q. suber* cork

Peak **4** was assigned to *p*-hydroxyphenyllactic acid (Figure 5.3), which shows a $[M-H]^-$ ion at *m/z* 181. Although there is no reference about the ESI fragmentation of this compound, its spectrum revealed the typical fragmentation of its constituents groups, with product ions at *m/z* 137 and 109, due to the losses of –COO and –CO groups,

respectively. The MS/MS spectrum and fragmentation pathway proposed for this compound is shown in Figure 5.4.

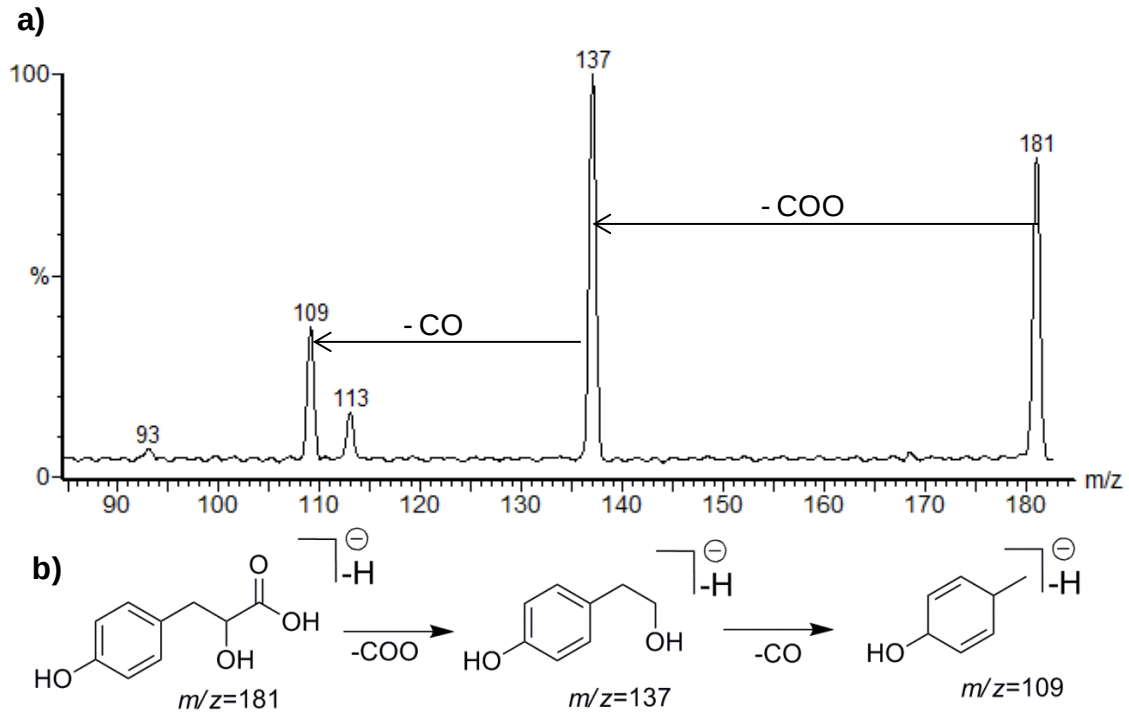


Figure 5.4 – a) MS/MS spectrum and b) fragmentation pathway proposed for *p*-hydroxyphenyllactic acid **4**

5.3.2.3 Coumarins

Compound **6** was identified as esculetin (Figure 5.5) based on its molecular ion $[M-H]^-$ at *m/z* 177 and the respective product ions at *m/z* 133 (−44Da, $-CO_2$) and 105 (−28Da, −CO), which are in agreement with the data published in literature [29].

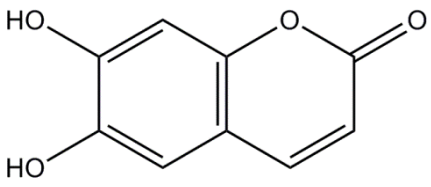


Figure 5.5 – Structure of esculetin **6**

5.3.2.4 Flavonoids

Eriodictyol **14** (Figure 5.6) has a molecular ion $[M-H]^-$ at *m/z* 287 and corresponding product ions at *m/z* 151 and 135 in agreement with published data [31]. The $[M-H]^-$

and the product ions of naringenin **15** (Figure 5.6) at *m/z* 271 and 177, 151, 119 and 107, respectively, are also in tune with the fragmentation suffered by the standard compound.

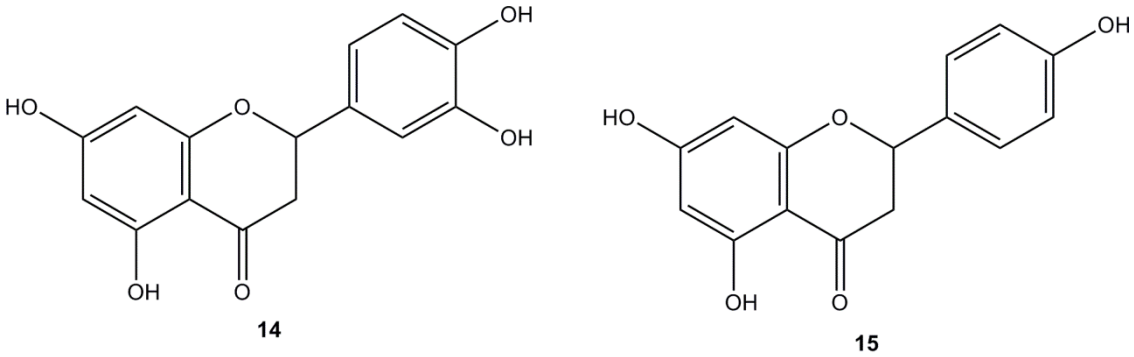


Figure 5.6 – Structures of flavonoids identified in *Q. suber* cork

Naringenin **15** is known to be present in citrus fruits [34]. However, its existence, together with eriodictyol **14**, was already reported in extracts of *Populus tremula* and in some pine species [35, 36].

5.3.3 HPLC quantification of phenolic compounds

The quantification of phenolic compounds by HPLC for each cork extract is presented in Table 5.2. The major components identified in all extracts were ellagic acid, followed by caffeic **7** and salicylic acids **13** in methanol/water extract, and by gallic **2** and protocatechuic **3** acids considering the methanol extraction followed by water. The contents of ellagic acid **12** are 2.0 g kg^{-1} of dry cork in MeOH/H_2O extract, and 1.6 and 0.5 g kg^{-1} of dry cork in MeOH and H_2O extracts, respectively; these values are in the range of those previously reported for natural cork extracts [12, 16], taking into consideration the well-known natural variability of cork composition [17].

In general, the phenolic components of the studied extracts were quantified in higher amounts considering the sum of MeOH and H_2O extracts (accounting for a total of 2.7 g kg^{-1}), than in the MeOH/H_2O extract (a total of 2.2 g kg^{-1}).

However, while the increase in the total amounts of phenolic compounds detected by HPLC–MS in MeOH and H_2O extracts (in mg kg^{-1} dry cork) is in the order of 21%, when compared to MeOH/H_2O extract, in the case of the corresponding total phenolic compounds contents (Table 5.1), the increase was considerably higher (roughly a sevenfold difference). This could means that a considerable fraction of phenolic compounds was not detected even by HPLC–MS under the experimental conditions

used. These should, certainly, correspond to high molecular weight cork tannins [13, 17]. In addition, some non-phenolic compounds, interfering in the Folin-Ciocalteu method and leading to an over-estimation of total phenolic compounds content [37] can contribute to the high values reported in both methanol and water extracts.

5.3.4 Antioxidant activity

Table 5.3 the antioxidant activity of the studied extracts, expressed in terms of the amount of extract required to reduce into 50% the DPPH concentration (IC_{50}), as well as in terms of the ascorbic acid equivalents (AAE) on a dry cork basis (mg AAE g^{-1} dry cork). The IC_{50} values for ascorbic acid and for BHT were also obtained and reported in Table 5.3 for comparative purposes. These extracts have revealed an antioxidant activity considerably higher than that reported for BHT and in the range of that measured for ascorbic acid. These observations are in agreement with reported results, which demonstrate that the antioxidant activity of some phenolic compounds could be higher than ascorbic acid and BHT [25, 38]. Interestingly, the antioxidant activities of the studied extracts are significantly higher than those reported for some wines, recognized for their antioxidant properties [39].

Table 5.3 – Antioxidant activity of the extracts of cork by DPPH radical scavenging, expressed as IC_{50} values, in µg of extract mL^{-1}, and as mg of ascorbic acid equivalents g^{-1} of dry cork

	IC_{50} (µg mL^{-1})	**values in ascorbic acid equivalents (mg AAE g^{-1} dry cork)**
Ascorbic acid in MeOH	2.12 ± 0.06	-
Ascorbic acid in H_2O	2.46 ± 0.11	-
BHT	18.79 ± 0.22	-
MeOH/H_2O extract	2.79 ± 0.15	9.15 ± 0.51
MeOH extract	3.58 ± 0.20	10.11 ± 0.54
H_2O extract	5.84 ± 0.29	15.59 ± 0.75

In general, the obtained IC_{50} values demonstrated a higher antioxidant activity for MeOH/H_2O extract, followed by MeOH and H_2O extracts. This decreasing antioxidant activity cannot be directly related with the amounts of components detected by HPLC–MS in each extract, as the decrease of this fraction (in mg g^{-1} of extract, see last row of Table 5.2) is much more substantial, nor with the total phenolic content (in g GAE g^{-1} of extract, Table 5.1), but rather, it should be the result of a combination of the effect of both the detected components along with the non-detected phenolic fractions.

Finally, the antioxidant capacity as AAE on a dry cork basis, increased proportionally to the extraction yields and total phenolic content (Table 5.1), with H_2O extract reaching the higher antioxidant potential. These results are promising, since cork water extracts could be used with obvious advantages as natural antioxidants in nutraceutical applications, when compared to organic solvent extracts.

5.4 Conclusions

In this work, different cork extracts from *Q. suber* L. were obtained following two distinct fractionation schemes, namely methanol/water extraction followed by ethyl ether fractionation and sequential extraction with methanol and water. The extracts were studied in terms of total phenolic content (Folin-Ciocalteu method), detailed composition by HPLC–MS, and antioxidant activity. The HPLC–MS allowed to identify 15 phenolic compounds, among which ellagic acid followed by gallic and protocatechuic acids were the most abundant, and several others were reported for the first time as cork components (namely salicylic acid, naringenin, eriodictyol, quinic acid and *p*-hydroxyphenyllactic acid). To the best of our knowledge, this is also the first paper describing the phenolic compounds present in a H_2O extract of cork. The antioxidant activity of the extracts, evaluated using the DPPH radical scavenging assay, showed to be considerably higher than that of BHT, and in the range of that of ascorbic acid. The antioxidant potential per mass unit of the three extracts is in similar ranges, but the high extraction yields obtained by water extraction constitutes a promising result for its exploitation in nutraceutical applications, as well for the valorisation of cork as a renewable resource.

References

[1] Kamm, B.; Kamm, M.; Gruber, P. R.; Kromus, S., Biorefinery Systems-An Overview. In *Biorefineries- Industrial Processes and Products: Status Quo and Future Directions*, Kamm, B.; Gruber, P.; Kamm, M., Eds. Wiley VCH: Weinheim, Germany, 2006; Vol. 1, pp 3-40.

[2] Fernando, S.; Adhikari, S.; Chandrapal, C.; Murali, N., Biorefineries: Current status, challenges, and future direction. *Energ. Fuel* **2006,** *20*, 1727-1737.

[3] Gandini, A.; Pascoal Neto, C.; Silvestre, A. J. D., Suberin: A promising renewable resource for novel macromolecular materials. *Prog. Polym. Sci.* **2006,** *31*, 878-892.

[4] Silva, S. P.; Sabino, M. A.; Fernandes, E. M.; Correlo, V. M.; Boesel, L. F.; Reis, R. L., Cork: properties, capabilities and applications. *Int. Mater. Rev.* **2005,** *50*, 345-365.

[5] Silvestre, A. J. D.; Neto, C. P.; Gandini, A., Cork and Suberins: Major Sources, Properties and Applications. In *Monomers, Polymers and Composites from Renewable Resources*, Mohamed Naceur, B.; Alessandro, G., Eds. Elsevier: Amsterdam, 2008; pp 305-320.

[6] APCOR Cork Statistics. http://www.apcor.pt/userfiles/File/Cork%20Statistics.pdf

[7] Gil, L., Cork powder waste: An overview. *Biomass Bioenerg.* **1997,** *13*, 59-61.

[8] Pereira, H., Chemical composition and variability of cork from *Quercus suber* L. *Wood Sci. Technol.* **1988,** *22*, 211-218.

[9] Pinto, P. C. R. O.; Sousa, A. R.; Silvestre, A. J. D.; Neto, C. P.; Gandini, A.; Eckerman, C.; Holmbom, B., Quercus suber and Betula pendula outer barks as renewable sources of oleochemicals: A comparative study. *Ind. Crop. Prod.* **2009,** *29*, 126-132.

[10] Sousa, A. F.; Gandini, A.; Silvestre, A. J. D.; Neto, C. P., Synthesis and Characterization of Novel Biopolyesters from Suberin and Model Comonomers. *Chemsuschem* **2008,** *1*, 1020-1025.

[11] Turley, A., Renewable chemicals A corking good polymer idea. *Chemistry & Industry* **2009**, 8-8.

[12] Sousa, A. F.; Pinto, P.; Silvestre, A. J. D.; Neto, C. P., Triterpenic and other lipophilic components from industrial cork byproducts. *J. Agric. Food Chem.* **2006,** *54*, 6888-6893.

[13] Pereira, H., Constituição química da cortiça - Estado actual dos conhecimentos. *Cortiça* **1979,** *483*, 259-264.

[14] Zimmermann, W.; Nimz, H.; Seemüller, E., ^{1}H and ^{13}C NMR spectroscopy study from corks of *Robus idaeus*, *Solanum tuberosum* and *Quercus suber*. *Holzforschung* **1985,** *39*, 45-49.

[15] García-Vallejo, M. C.; Conde, E.; Cadahia, E.; deSimon, B. F., Suberin composition of reproduction cork from Quercus suber. *Holzforschung* **1997,** *51*, 219-224.

[16] Conde, E.; Cadahía, E.; García-Vallejo, M. C.; de Simón, B. F.; Adrados, J. R. G., Low molecular weight polyphenols in cork of *Quercus suber*. *J. Agric. Food Chem.* **1997,** *45*, 2695-2700.

[17] Conde, E.; Cadahía, E.; García-Vallejo, M. C.; Fernández de Simón, B., Polyphenolic composition of *Quercus suber* cork from different Spanish provenances. *J. Agric. Food Chem.* **1998,** *46*, 3166-3171.

[18] Cadahia, E.; Conde, E.; de Simon, B. F.; Garcia-Vallejo, M. C., Changes in tannic composition of reproduction cork *Quercus suber* throughout industrial processing. *J. Agric. Food Chem.* **1998,** *46*, 2332-2336.

[19] Balasundram, N.; Sundram, K.; Samman, S., Phenolic compounds in plants and agri-industrial by-products: Antioxidant activity, occurrence, and potential uses. *Food Chem.* **2006,** *99*, 191-203.

[20] Noferi, M.; Masson, E.; Merlin, A.; Pizzi, A.; Deglise, X., Antioxidant characteristics of hydrolysable and polyflavonoid tannins: An ESR kinetics study. *J. Appl. Polym. Sci.* **1997,** *63*, 475-482.

[21] Proestos, C.; Boziaris, I. S.; Nychas, G. J. E.; Komaitis, M., Analysis of flavonoids and phenolic acids in Greek aromatic plants: Investigation of their antioxidant capacity and antimicrobial activity. *Food Chem.* **2006,** *95*, 664-671.

[22] Ito, N.; Hirose, M.; Fukushima, S.; Tsuda, H.; Shirai, T.; Tatematsu, M., Studies on antioxidants: Their carcinogenic and modifying effects on chemical carcinogenesis. *Food Chem. Toxicol.* **1986,** *24*, 1071-1082.

[23] Sharma, O. P.; Bhat, T. K., DPPH antioxidant assay revisited. *Food Chem.* **2009,** *113*, 1202-1205.

[24] Singleton, V. L.; Rossi, J. A., Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enol. Viticult.* **1965,** *16*, 144-158.

[25] Sanchez-Moreno, C.; Larrauri, J. A.; Saura-Calixto, F., A procedure to measure the antiradical efficiency of polyphenols. *J. Sci. Food Agric.* **1998,** *76*, 270-276.

[26] Kähkönen, M. P.; Hopia, A. I.; Vuorela, H. J.; Rauha, J.-P.; Pihlaja, K.; Kujala, T. S.; Heinonen, M., Antioxidant Activity of Plant Extracts Containing Phenolic Compounds. *J. Agric. Food Chem.* **1999,** *47*, 3954-3962.

[27] Mazzoleni, V.; Caldentey, P.; Silva, A., Phenolic compounds in cork used for production of wine stoppers as affected by storage and boiling of cork slabs. *Am. J. Enol. Viticult.* **1998,** *49*, 6-10.

[28] Ng, L. K.; Lafontaine, P.; Vanier, M., Characterization of ciggarette tobacco by direct electrospray ionization-ion trap mass spectrometry (ESI-ITMS) analyis of the aqueous extract - A novel and simple approach. *J. Agric. Food Chem.* **2004,** *52*, 7251-7257.

[29] Eyles, A.; Jones, W.; Riedl, K.; Cipollini, D.; Schwartz, S.; Chan, K.; Herms, D. A.; Bonello, P., Comparative phloem chemistry of manchurian (*Fraxinus mandshurica*) and two north american ash species (*Fraxinus americana* and *Fraxinus pennsylvanica*). *Journal of Chemical Ecology* **2007,** *33*, 1430-1448.

[30] Sanz, M.; Cadahia, E.; Esteruelas, E.; Munoz, A. M.; Simon, B. F.; Hernandez, T.; Estrella, I., Phenolic Compounds in Cherry (*Prunus avium*) Heartwood with a View to Their Use in Cooperage. *J. Agric. Food Chem.* **2010,** *58*, 4907-4914.

[31] Sudjaroen, Y.; Haubner, R.; Wurtele, G.; Hull, W. E.; Erben, G.; Spiegelhalder, B.; Changbumrung, S.; Bartsch, H.; Owen, R. W., Isolation and structure elucidation of phenolic antioxidants from Tamarind (Tamarindus indica L.) seeds and pericarp. *Food and Chemical Toxicology* **2005,** *43*, 1673-1682.

[32] Dufresne, C. J.; Farnworth, E. R., A review of latest research findings on the health promotion properties of tea. *Journal of Nutritional Biochemistry* **2001,** *12*, 404-421.

[33] Bianco, A.; Brufani, M.; Manna, F.; Melchioni, C., Synthesis of a carbocyclic sialic acid analogue for the inhibition of influenza virus neuraminidase. *Carbohydrate Research* **2001,** *332*, 23-31.

[34] de Lourdes Mata Bilbao, M.; Andres-Lacueva, C.; Jauregui, O.; Lamuela-Raventos, R. M., Determination of flavonoids in a Citrus fruit extract by LC-DAD and LC-MS. *Food Chemistry* **2007,** *101*, 1742-1747.

[35] Hartonen, K.; Parshintsev, J.; Sandberg, K.; Bergelin, E.; Nisula, L.; Riekkola, M.-L., Isolation of flavonoids from aspen knotwood by pressurized hot water extraction and comparison with other extraction techniques. *Talanta* **2007,** *74*, 32-38.

[36] Lantto, T. A.; Dorman, H. J. D.; Shikov, A. N.; Pozharitskaya, O. N.; Makarov, V. G.; Tikhonov, V. P.; Hiltunen, R.; Raasmaja, A., Chemical composition, antioxidative activity and cell viability effects of a Siberian pine (Pinus sibirica Du Tour) extract. *Food Chemistry* **2009,** *112*, 936-943.

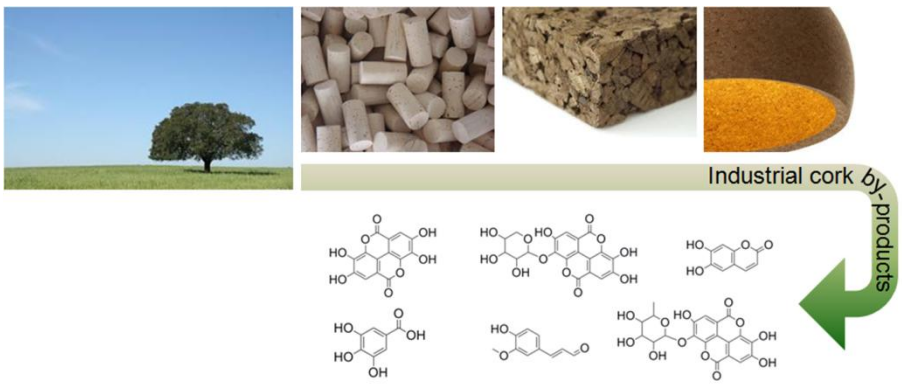
[37] Prior, R. L.; Wu, X. L.; Schaich, K., Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *J. Agric. Food Chem.* **2005,** *53*, 4290-4302.

[38] Cuvelier, M. E.; Richard, H.; Berset, C., Comparison of the antioxidative activity of some acid-phenols - structure activity relationship. *Bioscience Biotechnology and Biochemistry* **1992,** *56*, 324-325.

[39] Roussis, I. G.; Lambropoulos, I.; Tzimas, P.; Gkoulioti, A.; Marinos, V.; Tsoupeis, D.; Boutaris, I., Antioxidant activities of some Greek wines and wine phenolic extracts. *Journal of Food Composition and Analysis* **2008,** *21*, 614-621.

Part B

Phenolic compounds from industrial cork by-products



Adapted from:

Santos, S.A.O., Villaverde, J.J., Sousa, A.F., Coelho, J.F.J., Neto, C.P., Silvestre, A.J.D., Industrial cork by-products as a source of valuable phenolic compounds, *Industrial Crops and Products*, **submitted**

Abstract

The phenolic composition of cork powder and black condensate, two by-products from the cork industry, was investigated by the first time using high-performance liquid chromatography-multi-stage mass spectrometry (HPLC-MS^n). The same methodology of extraction was applied to cork, for comparative purposes. Eighteen phenolic compounds were identified in cork, five of them are reported for the first time as its constituents. Sixteen and thirteen phenolic compounds were identified as cork powder and black condensate components, respectively, with only one compound of each one published before as its constituent. The antioxidant scavenging of the extracts were evaluated, being, to our knowledge, the first study concerning the antioxidant activity of these cork residues. The extracts present an antioxidant activity higher than BHT, with black condensate showing the lowest IC_{50} value. These results, together with the phenolic content values, demonstrate the high potential of these residues as source of high value compounds

.

5.5 Introduction

Cork, the outer bark of *Quercus suber* L., an important species in the Mediterranean basin shows unique properties, such as high elasticity and low permeability [1-3]. This renewable material has a large variety of applications, among which the production of stoppers for wine and other alcoholic beverages is by far the most important, followed by its applications in thermal and/or acoustic insulation materials. Cork industry is a key sector in the Portuguese economy since the country is the main cork producer and exporter in the world (representing, in 2005, a value of 60% of the world cork exportations) [4].

During cork stoppers and disks production this industry produces cork residues, which are then granulated for the production of agglomerated materials. Throughout this process, a low granulometry fraction is generated, the “cork powder”, which is not suitable for the production of agglomerates. This by product represents about 22 % of the total cork production, corresponding annually to about 34 000 tonnes [5] and, due to its high heating value, is currently mainly used for energy production [5, 6].

The insulation corkboard industry also generates another residue, the “black condensate”, a pasty-like black waste, during the production of black agglomerates. This process involves the treatment of cork particles, without any adhesive and at high temperatures (250−500 °C), which cause the formation of vapours that condense later in autoclave pipes. Black condensate is removed periodically (2500 tonnes/year) and simply burned to produce energy [5].

Several studies have been carried out to study new applications for cork and its by-products [7, 8], aiming to promote their valorisation. In fact, the agro-forest by-products are seen, in the recent years, as emerging alternatives for the petrochemical derived products, within the emerging biorefinery concept [9]. Therefore, the detailed study of the chemical composition of cork and industrial cork by-products is a key step towards the implementation of these strategies.

Natural cork is mainly composed by suberin (~40%), lignin (~25%), polysaccharides (~20%) and extractives (~15%), with substantial variability, depending, among others, in climatic and soil conditions, the geographic origin or the tree dimensions and age [10-12]. Suberin, with its unique chemical composition and natural abundance, already has shown to be a promising valuable renewable resource in the preparation of novel materials [1, 3, 13-15]. Cork extractives have also attracted substantial attention. The

triterpenic fraction and friedelin in particular, due to their abundance and well know biological properties have attracted special attention [16-18]. The phenolic composition of cork from *Q. suber* has been object of a several studies in the last few years [12, 19-21]. Phenolic acids, aldehydes and flavonoids have been reported as cork constituents, with ellagic acid referred as the most abundant phenolic compound in cork extracts [10-12, 19]. These classes of compounds are well known by their numerous biological properties, such as antioxidant, anti-thrombotic or antiproliferative activities, among others [22, 23]. Several authors also reported cork as a valuable source of tannins [24], which are also known for their peculiar properties and applications [25, 26]. Recently, Fernandes *et al.* [21] also identified several gallic and ellagic acid derivatives in *Q. suber* cork.

Although there is a significant amount of studies focusing the composition of cork, there is a gap in the literature concerning the characterisation of industrial cork by-products. The aliphatic composition of cork powder and black condensate were already described [27]. However, studies concerning the characterisation of phenolic fraction from these industrial cork by-products are extremely scarce. In fact, only Sousa *et al.* [27] have identified some phenolic compounds in these residues, namely ellagic acid in cork powder and catechol, benzoic, ferulic and vanillic acids and two vanillic acid derivatives in black condensate. However, this study only involved analysis of extracts by gas chromatography-mass spectrometry (GC-MS), which do not allow the detection of more polar and higher molecular weight phenolic compounds.

In this way, and following our interest on the detailed characterisation and valorisation of cork by-products [13, 15, 27], the objective of the present study was to characterise in detail the phenolic fraction of cork powder and black condensate, as well as cork, for comparative purposes, using high-performance liquid chromatography-multi-stage mass spectrometry (HPLC-MS^n). Furthermore, the antioxidant scavenging of the extracts obtained were evaluated, being the first study concerning the analysis of this activity on these industrial cork by-products.

5.6 Materials and methods

5.6.1 Chemicals

Dichloromethane (99 % purity), gallic acid (purity higher than 97.5 %), quercetin (purity higher than 98 %), vanillin (99 % purity), Folin-Ciocalteu's phenol reagent and, 3,5-di-*tert*-4-butylhydroxytoluene (BHT) (purity higher than 99 %) and 2,2-diphenyl-1-

picrylhydrazyl hydrate (DPPH) were supplied by Sigma Chemical Co (Madrid, Spain). Protocatechuic acid (purity higher than 97 %), caffeic acid (purity higher than 95 %) and naringenin (98 % purity) were obtained from Aldrich Chemical Co (Madrid, Spain). HPLC-grade methanol, water and acetonitrile were supplied from Fisher Scientific Chemicals (Loures, Portugal). Sodium carbonate (99.9 % purity) was supplied by Pronalab (Lisbon, Portugal). Formic acid (purity higher than 98 %), ascorbic acid (purity higher than 99.5 %), methanol (purity higher than 99.8 %) and ellagic acid (96 % purity) were purchased from Fluka Chemie (Madrid, Spain). Solvents were filtered using a Solvent Filtration Apparatus 58061 from Supelco (Bellefonte, PA, USA).

5.6.2 Raw materials

Industrial cork powder (ICP) was sampled in Corticeira Amorim mill (Portugal); black condensate (BC) was sampled in Amorim Revestimentos mill (Portugal). *Q. suber* L. natural cork planks -“amadia” grade- (NC) was sampled from the south of Portugal (Herdade da Moinhola, Amorim Florestal mill, Portugal). Average samples of NC and BC were milled in a Retsch cross-beater mill SK1 (Haan, Germany), and the granulometric fraction of 40−60 mesh was used for analyses.

5.6.3 Sample preparation

About 20 g of each dried sample were submitted to a Soxhlet extraction with dichloromethane for 6 hours to remove the lipophilic fraction. The phenolic fraction was then extracted from the solid residues following previously optimised conditions [28, 29]: suspended (m/v: 1:100) in a methanol:water (MeOH:H_2O) mixture, 50/50 (v/v), at room temperature for 24 hours under constant stirring. The suspensions were then filtered, MeOH removed by low pressure evaporation and the extracts freeze dried.

5.6.4 Total phenolic content

The total phenolic content (TPC) of the extracts was determined by the Folin-Ciocalteu method [30], as described before [20]. The extracts concentrations ranged between 80 and 320 μg of extract mL^{-1}. TPC was calculated as gallic acid equivalents from the calibration curve of gallic acid standard solutions (10 - 85.0 μg mL^{-1}) and expressed as mg of gallic acid equivalents (GAE) g^{-1} of extract. The analyses were carried out in triplicate and the average value was calculated in each case.

5.6.5 HPLC-UV procedure

The HPLC system consisted of a variable loop Accela autosampler (200 vial capacity set at 15 °C), an Accela 600 LC pump and an Accela 80 Hz PDA detector (Thermo Fisher Scientific, San Jose, Ca, USA). The separation of the compounds was carried out with a gradient elution program at a flow rate of 0.2 mL min^{-1}, at 21 ºC, by using a Discovery® C-18 (15 cm x 2.1 mm x 5 µm) column supplied by Supelco (Agilent Technologies, Waldbronn, Germany). The injection volume in the HPLC system was 10 µl and the mobile phases consisted in water:acetonitrile (90:10, v/v) (A) and acetonitrile (B), both with 0.1% of formic acid. The following linear gradient was applied: 0-3 min: 0% B; 3-10 min: 0-10% B; 10-30 min: 10-20% B; 30-35 min: 20-25% B; 35-50 min: 25-50% B; 50-60 min: 50-0% B. Then the re-equilibration of the column was performed during 10 minutes before the next run. Double online detection was carried out in the diode array detector, at 280 and 340 nm, and UV spectra in a range of 200-600 nm were also recorded. Before the injection, each extract was dissolved in a MeOH:H_2O (50:50) mixture HPLC grade, to obtain final concentrations between 5 and 10 mg mL^{-1}, and then filtered through a 0.2 µm PTFE syringe filter.

5.6.6 ESI–MS^n analysis

The HPLC was coupled to a LCQ Fleet ion trap mass spectrometer (ThermoFinnigan, San Jose, CA, USA), equipped with an electrospray ionization source and operating in negative mode. The flow rate of nitrogen sheath and auxiliary gas were 40 and 5 (arbitrary units), respectively. The spray voltage was 5 kV and the capillary temperature, 300 °C. The capillary and tune lens voltages were set at -28 V and -115 V, respectively. CID-MS^n experiments were performed on mass-selected precursor ions in the range of *m*/*z* 100–1000. The isolation width of precursor ions was 1.0 mass units. The scan time was equal to 100 ms and the collision energy was optimised between 15-45 (arbitrary units), using helium as collision gas. The data acquisition was carried out by using Xcalibur® data system (ThermoFinnigan, San Jose, CA, USA).

5.6.7 HPLC-UV quantification

Calibration curves were obtained by HPLC injection of gallic, protocatechuic, caffeic and ellagic acids and vanillin, quercetin and naringenin standard solutions in MeOH, with five different concentrations between 5 and 500 µg mL^{-1}. The data relevant for obtaining the calibration curves is shown in Table 5.4.

Table 5.4 – Calibration data used for the HPLC-UV quantification of phenolic components of cork, cork powder and black condensate extracts

Compound	λ (nm)	Conc. Range (μg mL^{-1})	Calibration curve[a]	R^2	LOQ[b]	LOD[b]
Gallic acid	280	5 –200	y = 240930x-82282	0.999	10.68	35.59
Protocatechuic acid	280	5 – 500	y = 289949x+1523054	1.000	12.20	40.66
Vanillin	280	5 – 200	Y = 526547x+590233	0.993	24.16	80.53
Caffeic acid	280	5 – 200	Y = 651735x+893078	0.991	28.05	93.52
Ellagic acid	340	5 – 500	y = 229614x-1091693	0.999	45.94	153.13
Quercetin	340	5 – 250	y = 519503x-1910957	0.998	32.50	108.34
Naringenin	280	5 – 200	y = 623591x+1103161	0.997	38.81	129.36

[a] y = peak area, x = concentration in μg mL^{-1}; [b] expressed in μg mL^{-1}

Quantification of individual compounds (Table 5.7) was obtained using the calibration data of the most similar standard, as for some of which no pure reference compounds were available. Compounds concentrations were calculated in triplicate and the mean value calculated in each case.

5.6.8 Antioxidant activity

The antioxidant activity of the extracts was determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging [31], following a procedure described before [20]. The extracts concentrations were between 1 and 7 μg mL^{-1}. Ascorbic acid and 3,5-Di-*tert*-4-butylhydroxytoluene (BHT) were used as reference compounds.

Triplicate measurements were carried out. The antioxidant activity expressed as IC_{50} values and also as g of ascorbic acid equivalents kg^{-1} of dry basis (g AAE Kg^{-1} of dry starting material).

5.7 Results and discussion

5.7.1 Extraction yields and total phenolic content

The extraction yields of the MeOH/H_2O extracts of the cork and cork residues analysed and the respective total phenolic content, determined by Folin-Ciocalteu method are shown in Table 5.5.

The extraction yields are distinct between them, with NC showing the higher value, followed by ICP and BC (about 5.9, 2.3 and 1 %, respectively). These yields are slightly higher than those previously reported [20, 27], which, apart from natural variation of the composition of these materials, is also associated with the extraction conditions used, including the solvent mixtures and the higher m/v ratio used. Black condensate shows

the lower extraction yield value (9.9 g Kg^{-1} dry starting material), which is due to the volatile and predominantly dichloromethane nature soluble (~93%) of this residue [27].

Table 5.5 – Extraction yield and total phenolic content of cork, cork powder and black condensate

Extract	Extraction yield (%)	Total phenolic content	
		(mg GAE g^{-1} of extract)	(g GAE Kg^{-1} of dry starting material)
NC	5.93	336.34 ± 1.15	19.94 ± 0.07
ICP	2.26	254.54 ± 0.28	5.74 ± 0.01
BC	0.99	167.32 ± 0.61	1.66 ± 0.01

The total phenolic content of the three extracts ranged between 167.32 – 336.34 mg GAE g^{-1} of extract. The NC extract shows the highest value, followed by ICP and BC. This was expected since, on the one hand, cork powder is composed mainly by the inner and by the unsheltered outer fractions of cork planks [27], which could lead to the degradation of phenolic compounds. On the other hand, black condensate is mainly composed by more volatile compounds. When expressed on g GAE Kg^{-1} of starting material, the difference between the extracts becomes more explicit, as a result of the extraction yield values. The NC extract shows a higher value than those published before, but obtained using other solid-liquid extraction methodologies [12, 20, 24], which demonstrates the suitability of a methanol:water extraction applied at mild conditions in the extraction of phenolic compounds. Nevertheless, the three extracts show total phenolic contents in the same range of those published before for a wide range of fruits and plants [32], well-known as sources of this class of compounds. Moreover, NC shows a value slightly higher than those.

5.7.2 Identification of phenolic compounds

The identification of the components of MeOH/H_2O extracts of NC, ICP and BC was carried out by HPLC-UV-MS^n. Table 5.6 summarizes the phenolic compounds identified in each extract, their retention time, the molecular ion $[M-H]^-$, and the product ions obtained by MS^n. Table 5.6 also contains information about which compounds were already described as cork constituents, and the respective bibliographic reference.

Table 5.6 – Phenolic compounds identified in cork, cork powder and black condensate and corresponding MS^n fragmentation profiles

No.	Rt (min)	Compound	$[M-H]^-$ (*m/z*)	MS^n product ions (*m/z*)	identified
1	2.72	Quinic acid	191	MS^2: 173, 171, 127, 111, 93, 85	
2	2.91	Gallic acid	169	MS^2: 125	Co
3	4.52	*p*-hydroxyphenyllactic acid	181	MS^2: 137, 113, 109	
4	5.05	Protocatechuic acid	153	MS^2: 109	Co
5	8.55	*p*-coumaric acid	163	MS^2: 119	Co
6	9.57	Methyl gallate	183	MS^2: 168, 124	[28]
7	10.27	Vanillin	151	MS^2: 136	Co
8	11.38	Esculetin	177	MS^2: 133, 105	[20]
9	11.60	Brevifolin-carboxylic acid	291	MS^2: 247; MS^3: 203, 175	[33]
10	11.68	Caffeic acid	179	MS^2: 135	Co
11	13.31	Coniferaldehyde	177	MS^2: 162, 149, 133	[21]
12	15.40	Caffeic acid isoprenyl ester	247	MS^2: 179; MS^3: 135	[34]
13	14.37	Valoneic acid dilactone	469	MS^2: 425; MS^3: 407, 301	[21]
14	16.80	Ellagic acid-pentoside	433	MS^2: **301**, 300; MS^2: 284, 257, 229, 228, 185	[35]
15	16.91	Ellagic acid-rhamnoside	447	MS^2: **301**, 300; MS^3: 257, 229, 185	[35]
16	17.04	Ellagic acid	301	MS^2: 284, 257, 229, 213, 201, 185	Co
17	19.74	Isorhamnetin-rhamnoside	461	MS^2: **315**, 300; MS^3: 300; MS^4: 272, 271, 244	[28]
18	20.42	Eriodictyol	287	MS^2: 151	[20]
19	21.92	Isorhamnetin	315	MS^2: 300; MS^3: 272, 271, 244	[28]
20	22.62	Ferulic acid	193	MS^2: 178, 149, 134	Co

5.7.2.1 Phenolic acids, aldehydes and derivatives

Gallic **2** and protocatechuic **4** acids and vanillin **7** (Figure 5.7) were identified by comparing their retention times and mass spectra with those of reference substances. Compound **1** was identified as quinic acid (Figure 5.7), based on its $[M-H]^-$ at *m/z* 191 and MS^2 spectrum showing ions at *m/z* 173 ($[M-H-H_2O]^-$) and 127 ($[M-H-CO-2H_2O]^-$) [20]. Compound **3** was assigned to *p*-hydroxyphenyllactic acid, with its $[M-H]^-$ ion at *m/z* 181 showing product ions at *m/z* 137 ($[M-H-COO]^-$) and at *m/z* 109 ($[M-H-COO-CO]^-$) [20]. Compound **6** was identified as methyl gallate (figure 5.7) based on its $[M-H]^-$ ion at *m/z* 183 and MS^2 spectrum showing the product ions at *m/z* 168 (-15 Da, $-CH_3$) and at *m/z* 124 (further loss of carboxylic group) [28].

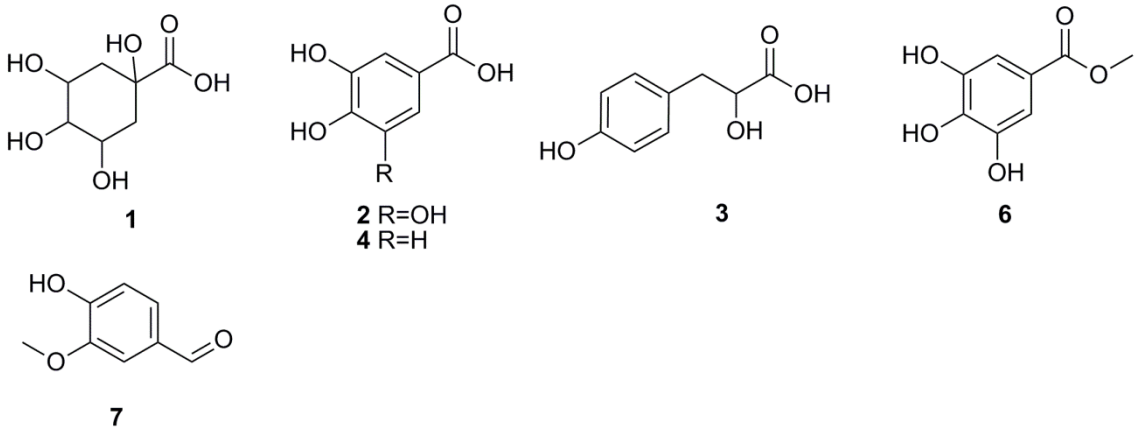


Figure 5.7 – Structures of phenolic acids, aldehydes and derivatives identified in *Q. suber* cork, cork powder and black condensate

5.7.2.2 Cinnamic acids and derivatives

Compounds **10** and **20** were identified as caffeic and ferulic acids (Figure 5.8), respectively, based on their retention times, $[M-H]^-$ ions and resultant product ions, which match with the respective reference compounds. Compound **11** presents a $[M-H]^-$ ion at *m/z* 177 and respective MS^2 spectrum characteristic of coniferaldehyde [21], with product ions at *m/z* 162 ($[M-H-CH_3]^-$) and at *m/z* 149 ($[M-H-CO]^-$).

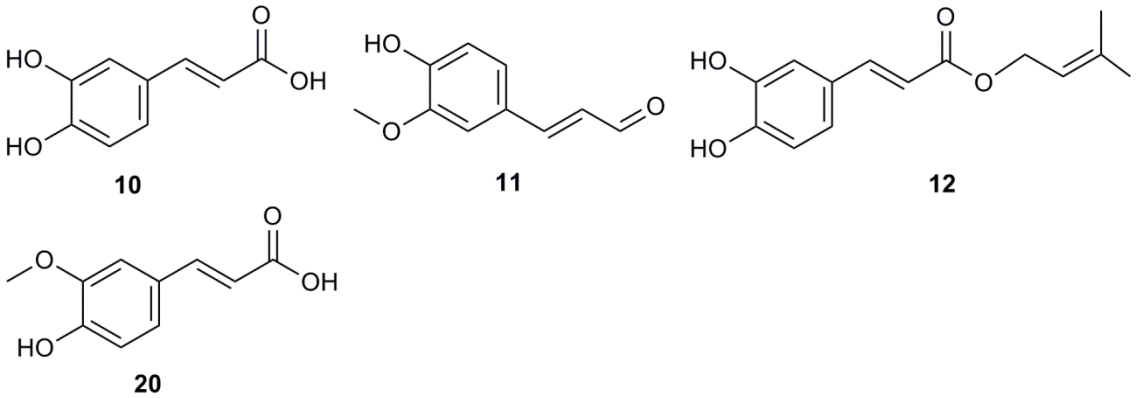


Figure 5.8 – Structures of cinnamic acids and derivatives identified in *Q. suber* cork, cork powder and black condensate

Compound **12** was assigned to caffeic acid isoprenyl ester, based on its $[M-H]^-$ ion at *m/z* 247 and further MS^2 spectrum, which has a product ion at *m/z* 179, corresponding to the phenolic acid (Figure 5.9). Further MS^3 spectrum (Figure 5.9) of this ion shows the characteristic fragmentation of caffeic acid authentic standard, with a product ion at *m/z* 135, resulting from the loss of the carboxylic group [34].

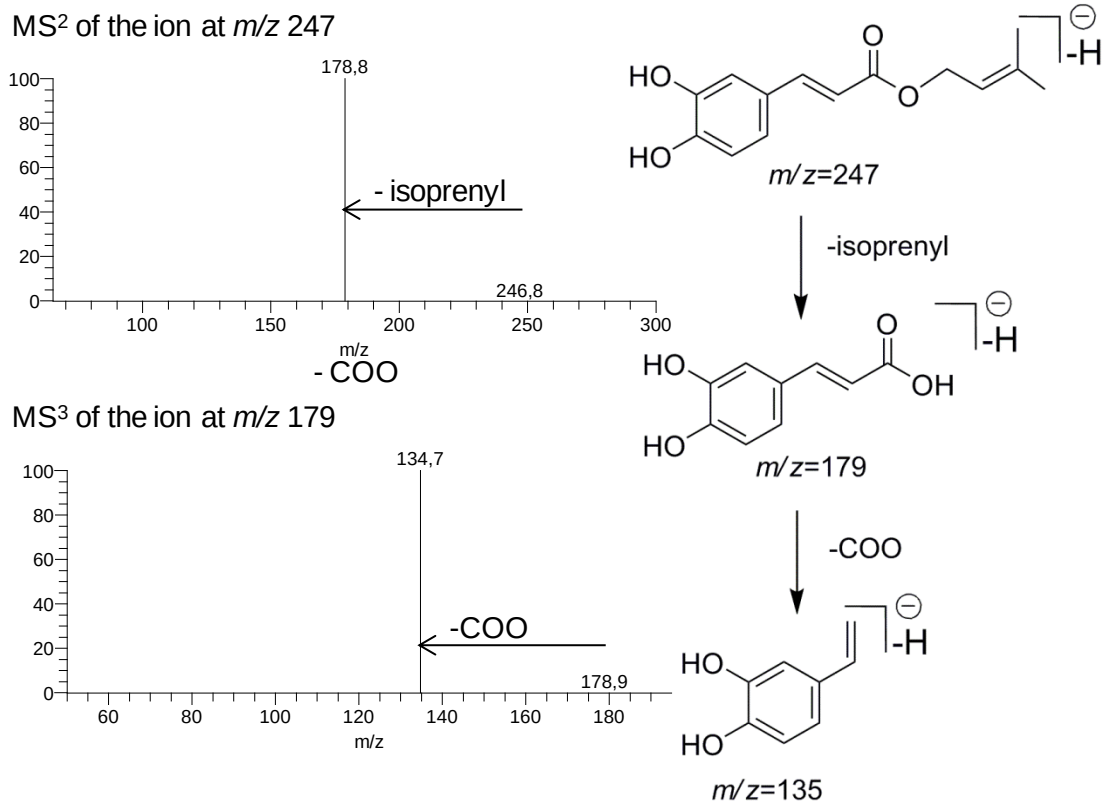


Figure 5.9 – MS^n spectra (left) and fragmentation pathway (right) of caffeic acid isoprenyl ester **12**

5.7.2.3 Coumarins and derivatives

Compound **8** was identified as esculetin, on the basis of its characteristic $[M-H]^-$ at *m/z* 177 and MS^2 product ions at *m/z* 133 and at *m/z* 105, due to the loss of –COO and further loss of –CO groups, respectively [20].

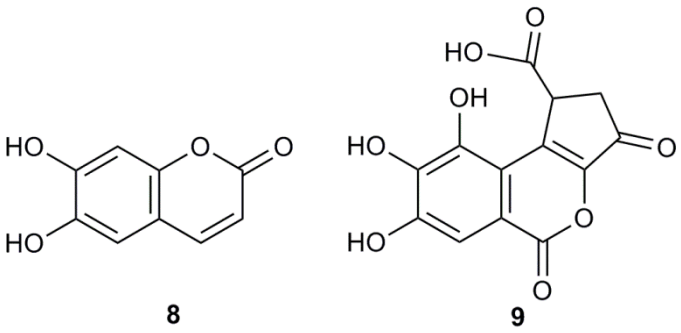


Figure 5.10 – Structures of coumarins identified in *Q. suber* cork, cork powder and black condensate

Compound **9** was assigned to brevifolin carboxylic acid, with its $[M-H]^-$ ion at *m/z* 291 and MS^2 and MS^3 spectra (Figure 5.11), which show the product ions at *m/z* 247 (-44 Da, -COO), at *m/z* 203 (-44 Da, -COO) and at *m/z* 175 (-28 Da, -CO). These data are consistent with those reported in the literature [33].

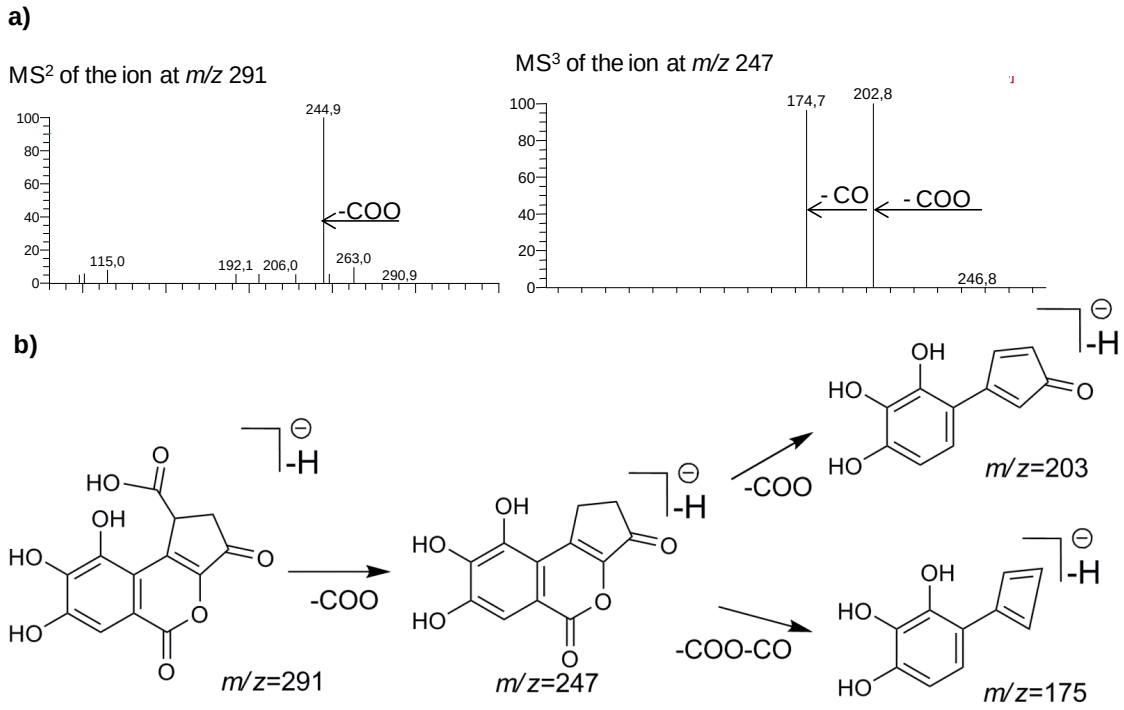


Figure 5.11 – a) MS^n spectra and b) fragmentation pathway of brevifolin carboxylic acid **9**

5.7.2.4 Ellagic acid and derivatives

Compound **16** was assigned to ellagic acid (Figure 5.12), based on its retention time, $[M-H]^-$ ion at *m/z* 301 and respective product ions, which match with those of reference compound. Compound **13** was identified as valoneic acid dilactone (Figure 5.12), on the basis of its $[M-H]^-$ ion at *m/z* 469 and MS^2 product ion at *m/z* 425 (-44 Da, -COO). This identification was further corroborated with the MS^3 spectrum of this last ion, showing the product ions at *m/z* 407 (-18 Da, $-H_2O$) and at *m/z* 301, corresponding to the ellagic acid moiety [21]. Compounds **14** and **15** were identified as ellagic acid pentoside and ellagic acid rhamnoside (Figure 5.12), respectively, based on their characteristic molecular ion $[M-H]^-$, at *m/z* 433 and 477, respectively, and MS^n fragmentation profile. Both compounds show a MS^2 product ion at *m/z* 301, corresponding respectively to the loss of a pentose (compound **14**) and rhamnose (compound **15**) units [29, 35]. Furthermore, the MS^3 spectra of the ions at *m/z* 301 show the characteristic fragmentation profile of ellagic acid standard.

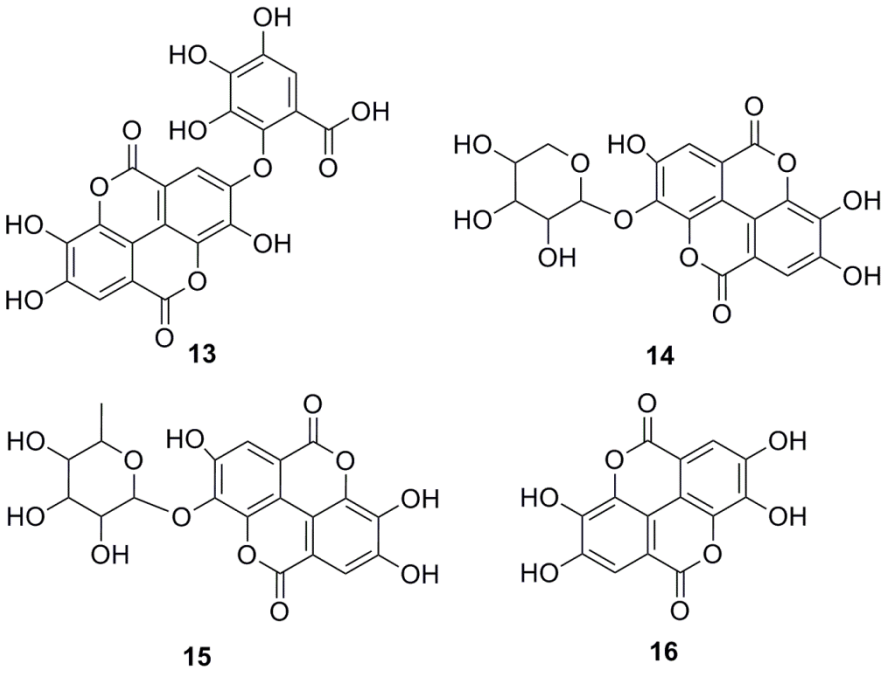


Figure 5.12 – Structures of ellagic acids and derivatives identified in *Q. suber* cork, cork powder and black condensate

5.7.2.5 Flavonoids and flavonoid glycosides

Compound **17** was assigned to isorhamnetin-rhamnoside (Figure 5.13), with its $[M-H]^-$ at *m/z* 461 and MS^2 spectrum showing a product ion at *m/z* 315 (loss of rhamnose moiety). Additionally, the subsequent MS^3 (315→300) and MS^4 (300→272) product ions are in agreement with those previously published for isorhamnetin aglycone [28].

In addition, compound **19** was identified as isorhamnetin (Figure 5.13), with its $[M-H]^-$ at *m/z* 315 and its MS^n product ions matching with this *O*-methylated-flavonol [28]. Compound **18** was assigned to eriodictyol (Figure 5.13), based on its $[M-H]^-$ ion at *m/z* 287 and MS^2 product ion at *m/z* 151, corresponding to retro-Diels-Alder fission $^{1,3}A^-$, characteristic of this flavanone [28, 36].

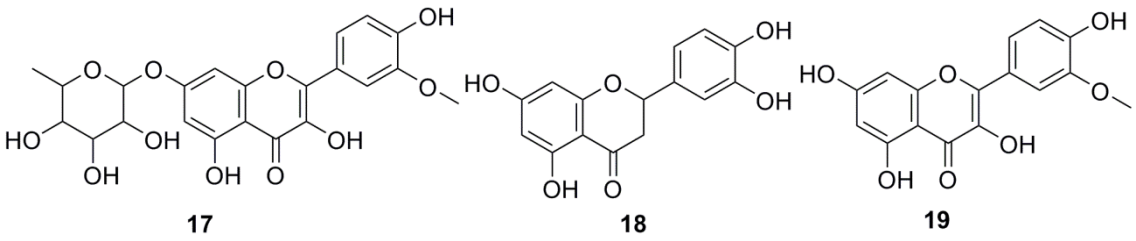


Figure 5.13 – Flavonoids and flavonoid glycosides identified in *Q. suber* cork, cork powder and black condensate

5.7.3 HPLC quantification of phenolic compounds

The phenolic composition of each extract quantified by HPLC is given in Table 5.7.

Most of the compounds identified in the present extracts were previously reported as *Q. suber* cork components, namely quinic **1**, gallic **2**, *p*-hydroxypheyllactic **3**, protocatechuic **4**, *p*-coumaric **5**, caffeic **10**, ellagic **16** and ferulic **20** acids and vanillin **7**, esculetin **8**, coniferaldehyde **11**, valoneic acid dilactone **13**, ellagic acid-pentoside **14**, ellagic acid-rhamnoside **15** and eriodictyol **18** [12, 19-21, 37]. Additionally, five phenolic compounds are reported here for the first time as *Quercus suber* cork constituents, namely methyl gallate **6**, brevifolin carboxylic acid **9**, caffeic acid isoprenyl ester **12** isorhamnetin-rhamnoside **17** and isorhamnetin **19**. Some of these compounds are well known as constituents of other vegetal sources [28, 29, 33, 34]. Ellagic **16** and ferulic **20** acids were also identified before as constituents of cork powder and black condensate, respectively [27]. Therefore, fifteen of the sixteen phenolic compounds identified are reported for the first time as constituents of industrial cork powder, namely quinic **1**, gallic **2**, protocatechuic **4**, caffeic **10** and ferulic **20** acids and methyl gallate **6**, esculetin **8**, brevifolin carboxylic acid **9**, coniferaldehyde **11**, caffeic acid isoprenyl ester **12**, valoneic acid dilactone **13**, ellagic acid-pentoside **14**, ellagic acid-rhamnoside **15**, isorhamnetin-rhamnoside **17** and isorhamnetin **19**. In the same way, thirteen phenolic compounds were identified in black condensate, twelve of them for the first time, namely quinic **1**, gallic **2**, *p*-hydroxypheyllactic **3**, protocatechuic **4**, *p*-coumaric **5**, caffeic **10** and ellagic **16** acids and vanillin **7**, esculetin **8**, coniferaldehyde

11, caffeic acid isoprenyl ester **12** and eriodictyol **18**. From a qualitative point of view, cork and cork powder were found to have a similar composition. With the exception of *p*-hydroxyphenyllactic acid **3** and eriodictyol **18** (identified in NC extract), all the other compounds were identified in both extracts.

Ellagic acid **16** was identified as the major phenolic compound in cork and cork powder extracts. In fact, this compound has been widely reported as the main constituent of polar extracts of cork from *Q. suber* [12, 20]. However, the contents of this compound differ significantly, accounting for 1246.46 mg Kg^{-1} dry cork against 527.59 mg Kg^{-1} dry cork powder. A similar difference in the relative abundance of components from cork and cork powder was already reported before for triterpenic compounds [27] and must be related to the variability of cork composition. In the rank of the major compounds it follows ellagic acid-pentoside **14** and ellagic acid-rhamnoside **15** and gallic acid **2** in NC extract and gallic acid **2** and esculetin **8** in ICP extract. In contrast, coniferaldehyde **11**, esculetin **8** and gallic acid **2** were identified as the main constituents of black condensate, with values of 194.34, 125.28 and 118.46 mg Kg^{-1} dry black condensate, respectively.

Cork has shown considerably higher content on identified phenolic compounds (4.2 g Kg^{-1} dry basis), which is in agreement with the total phenolic content analysis by Folin-Ciocalteu method, described above. Furthermore, the amount of identified phenolic compounds of NC is considerably higher than those described before for cork extracts [20], which is due, mainly to the higher extraction yield obtained in this study. When the phenolic content is expressed as mg g^{-1} of extract the differences between the three extracts remain almost imperceptible, with the BC extract with the higher amount of identified phenolic compounds. This result demonstrates the enormous potential of this unexploited residue together with cork powder as a source of valuable phenolic compounds.

.

Table 5.7 – Abundance of phenolic compounds identified in cork, cork powder and black condensate

No.	Compound	λ (nm)	Rt (min)	Phenolic content (mg Kg^{-1} dry starting material)			presence in cork or cork by-products
				NC	ICP	BC	
1	Quinic acid[a]	280	2.72	372.86±1.94	137.02±0.50	117.17±0.30	NC [20]
2	Gallic acid[a]	280	2.91	736.48±1.63	263.04±0.52	118.46±0.61	NC [12, 19-21, 37]
3	*p*-hydroxyphenyllactic acid[a]	280	4.52	TR	-	49.36±0.12	NC [20]
4	Protocatechuic acid[b]	280	5.05	79.26±0.10	16.44±0.01	9.97±0.03	NC [12, 19-21, 37]
5	*p*-coumaric acid[c]	280	8.55	-	-	35.76±0.22	NC [20]
6	Methyl gallate[a]	280	9.57	251.43±0.06	96.93±0.56	-	RP[j]
7	Vanillin[d]	280	10.27	-	-	32.47±0.25	NC [12, 19, 21, 37]
8	Esculetin[a]	280	11.38	391.59±1.10	176.80±0.60	125.28±0.65	NC [20]
9	Brevifolin-carboxylic acid[a]	280	11.60	102.03±0.08[i(9+10)]	53.72±0.15[i(9+10)]	-	RP
10	Caffeic acid[c]	280	11.68			17.68±0.05	NC [12, 19-21, 37]
11	Coniferaldehyde[a]	280	13.31	TR[h]	TR	194.34±0.56	NC [12, 19, 21, 37]
12	Caffeic acid isoprenyl ester[c]	280	15.40	127.98±0.28	82.47±0.29	13.32±0.04	RP
13	Valoneic acid dilactone[e]	340	14.37	168.01±0.70	46.05±0.11	-	NC [21, 37]
14	Ellagic acid-pentoside[e]	340	16.80	770.16±0.15[i(14+15)]	46.18±0.15[i(14+15)]	-	NC [37]
15	Ellagic acid-rhamnoside[e]	340	16.91			-	NC [37]
16	Ellagic acid[e]	340	17.04	1246.46±0.18	527.59±1.70	52.52±0.18	NC [12, 19-21, 37]; ICP [27]
17	Isorhamnetin-rhamnoside[f]	340	19.74	TR	TR	-	RP
18	Eriodictyol[g]	280	20.42	TR	-	TR	NC [20]
19	Isorhamnetin[f]	340	21.92	TR	TR	-	RP
20	Ferulic acid[c]	280	22.62	TR	14.77±0.02	TR	NC [12, 19-21, 37]; BC [27]
	Total (mg Kg^{-1} dry starting material)			**4246.28±5.35**	**1461.01±0.79**	**766.73±0.93**	
	Total (mg g^{-1} extract)			**71.63±0.09**	**64.73±0.04**	**77.51±0.09**	

Calibrations curve used: [a] gallic acid, [b] protocatechuic acid, [c] caffeic acid, [d] vanillin, [e] ellagic acid, [f] quercetin, [g] naringenin; [h] TR – traces; [i]Sum of the phenolic content by partial overlapping; [j] reported for the first time as *Quercus suber* cork component.

5.7.4 Antioxidant activity

Table 5.8 presents the results of the antioxidant activity of the studied extracts, expressed in terms of the amount of extract needed to decrease the DPPH concentration by 50% (IC_{50}), as well as in terms of the ascorbic acid equivalents (AAE) (mg AAE Kg^{-1} of dry starting material). The IC_{50} values for ascorbic acid and for BHT were also obtained and reported in Table 5.8 for comparative purposes. It is worth mentioning that this is the first study reporting the antioxidant activity of industry residues ICP and BC, shedding some light into the potential future applications.

Table 5.8 – Antioxidant activity of the extracts of cork, cork powder and black condensate by DPPH radical scavenging

	IC_{50} (μg mL^{-1})	**values in ascorbic acid equivalents (mg AAE g^{-1} dry starting material)**
Ascorbic acid	2.12 ± 0.02	-
BHT	18.79 ± 0.22	-
NC	4.77 ± 0.02	26.29 ± 0.10
ICP	3.33 ± 0.02	14.36 ± 0.07
BC	1.57 ± 0.01	13.34 ± 0.08

Comparing the antioxidant activity of the three extracts expressed as mg AAE Kg^{-1} of dry starting material, the three extracts present trends similar to those presented for phenolic content: NC has the highest antioxidant scavenging, followed by ICP and by BC, in line with the amounts of phenolic compounds detected by HPLC-MS and with the reported total phenolic content values.

The present extracts have revealed an antioxidant activity considerably higher than that for BHT and in some range of ascorbic acid. Furthermore, the IC_{50} values are in the same range of those reported before for *Quercus suber* cork extracts [20]. Interestingly, black condensate extract shows the highest antioxidant activity, even higher than ascorbic acid. This result, together with the phenolic composition, shows that BC is a promising source of valuable phenolic compounds. The development of methodologies to isolate and purify those compounds/fractions will constitute a relevant contribution to the valorisation of cork residues, instead of simply burning them, as a renewable resource.

5.8 Conclusions

To our knowledge, the phenolic composition of cork powder and black condensate, two by-products from the cork industry, was investigated for the first time by using HPLC-MS. To comparative purposes, the same methodology of extraction was applied to cork. Eighteen phenolic compounds were identified in cork, five of them are reported for the first time as its constituents, namely methyl gallate, brevifolin carboxylic acid, caffeic acid isoprenyl ester, isorhamnetin-rhamnoside and isorhamnetin. Sixteen and thirteen phenolic compounds were identified as cork powder and black condensate components, respectively, with only one compound of each one published before as its constituent. Ellagic acid is the major compound in both cork and cork powder extracts, followed by ellagic acid-pentoside, ellagic-acid rhamnoside and gallic acid in cork and by gallic acid and esculetin in cork powder Coniferaldehyde, esculetin and gallic acid are the major compounds of black condensate. The antioxidant scavenging of the extracts were evaluated, being, to our knowledge, the first study concerning the antioxidant activity of these cork residues. The extracts present an antioxidant activity higher than BHT, with black condensate showing the lowest IC_{50} value. These results, together with the phenolic content values, demonstrate the potential of these residues as a source of high value compounds

References

[1] Silvestre, A. J. D.; Neto, C. P.; Gandini, A., Cork and Suberins: Major Sources, Properties and Applications. In *Monomers, Polymers and Composites from Renewable Resources*, Mohamed Naceur, B.; Alessandro, G., Eds. Elsevier: Amsterdam, 2008; pp 305-320.

[2] Silva, S. P.; Sabino, M. A.; Fernandes, E. M.; Correlo, V. M.; Boesel, L. F.; Reis, R. L., Cork: properties, capabilities and applications. *Int. Mater. Rev.* **2005,** *50*, 345-365.

[3] Gandini, A.; Pascoal Neto, C.; Silvestre, A. J. D., Suberin: A promising renewable resource for novel macromolecular materials. *Prog. Polym. Sci.* **2006,** *31*, 878-892.

[4] APCOR Cork Statistics. http://www.apcor.pt/userfiles/File/Cork%20Statistics.pdf (September, 2012)

[5] Gil, L., *Cortiça: Produção Tecnologia e Aplicação*. INETI, Lisbon, Portugal: 1996.

[6] Gil, L., Cork powder waste: An overview. *Biomass Bioenerg.* **1997,** *13*, 59-61.

[7] Pintor, A. M. A.; Ferreira, C. I. A.; Pereira, J. C.; Correia, P.; Silva, S. P.; Vilar, V. J. P.; Botelho, C. M. S.; Boaventura, R. A. R., Use of cork powder and granules for the adsorption of pollutants: A review. *Water Res.* **2012,** *46*, 3152-3166.

[8] Pires, R. A.; Mano, J. F.; Reis, R. L., Surface properties of extracts from cork black condensate. *Holzforschung* **2010,** *64*, 217-222.

[9] Kamm, B.; Kamm, M.; Gruber, P. R.; Kromus, S., Biorefinery Systems-An Overview. In *Biorefineries- Industrial Processes and Products: Status Quo and Future Directions*, Kamm, B.; Gruber, P.; Kamm, M., Eds. Wiley VCH: Weinheim, Germany, 2006; Vol. 1, pp 3-40.

[10] Pereira, H., Chemical composition and variability of cork from *Quercus suber* L. *Wood Sci. Technol.* **1988,** *22*, 211-218.

[11] Conde, E.; Cadahia, E.; Garcia-Vallejo, M. C.; González Adrados, J. R., Chemical characterization of reproduction cork from Spanish *Quercus suber*. *J. Wood Chem. Technol.* **1998,** *18*, 447-469.

[12] Conde, E.; Cadahía, E.; García-Vallejo, M. C.; Fernández de Simón, B., Polyphenolic composition of *Quercus suber* cork from different Spanish provenances. *J. Agric. Food Chem.* **1998,** *46*, 3166-3171.

[13] Sousa, A. F.; Gandini, A.; Silvestre, A. J. D.; Neto, C. P., Synthesis and Characterization of Novel Biopolyesters from Suberin and Model Comonomers. *Chemsuschem* **2008,** *1*, 1020-1025.

[14] Sousa, A. F.; Gandini, A.; Silvestre, A. J. D.; Pascoal Neto, C., Determination of the Hydroxy and Carboxylic Acid Groups in Natural Complex Mixtures of Hydroxy Fatty Acids by H-1 Nuclear Magnetic Resonance Spectroscopy. *Appl. Spectrosc.* **2009,** *63*, 873-878.

[15] Sousa, A. F.; Gandini, A.; Silvestre, A. J. D.; Pascoal Neto, C.; Cruz Pinto, J. J. C.; Eckerman, C.; Holmbom, B., Novel Suberin-Based Biopolyesters: From Synthesis to Properties. *J. Polym. Sci. Pol. Chem.* **2011,** *49*, 2281-2291.

[16] Moiteiro, C.; Curto, M. J. M.; Mohamed, N.; Bailen, M.; Martinez-Diaz, R.; Gonzalez-Coloma, A., Biovalorization of friedelane triterpenes derived from cork processing industry byproducts. *J. Agric. Food Chem.* **2006,** *54*, 3566-3571.

[17] Coquet, C.; Ferre, E.; Peyronel, D.; Dal Farra, C.; Farnet, A. M., Identification of new molecules extracted from *Quercus suber* L. cork. *C. R. Biol.* **2008,** *331*, 853-858.

[18] Castola, V.; Bighelli, A.; Rezzi, S.; Melloni, G.; Gladiali, S.; Desjobert, J.-M.; Casanova, J., Composition and chemical variability of the triterpene fraction of dichloromethane extracts of cork (*Quercus suber* L.). *Ind. Crop. Prod.* **2002,** *15*, 15-22.

[19] Conde, E.; Cadahía, E.; García-Vallejo, M. C.; de Simón, B. F.; Adrados, J. R. G., Low molecular weight polyphenols in cork of *Quercus suber*. *J. Agric. Food Chem.* **1997,** *45*, 2695-2700.

[20] Santos, S. A. O.; Pinto, P. C. R. O.; Silvestre, A. J. D.; Neto, C. P., Chemical composition and antioxidant activity of phenolic extracts of cork from *Quercus suber* L. *Ind. Crop. Prod.* **2010,** *31*, 521-526.

[21] Fernandes, A.; Sousa, A.; Mateus, N.; Cabral, M.; de Freitas, V., Analysis of phenolic compounds in cork from *Quercus suber* L. by HPLC–DAD/ESI–MS. *Food Chem.* **2011,** *125*, 1398-1405.

[22] RiceEvans, C. A.; Miller, J.; Paganga, G., Antioxidant properties of phenolic compounds. *Trends Plant Sci.* **1997,** *2*, 152-159.

[23] Kris-Etherton, P. M.; Hecker, K. D.; Bonanome, A.; Coval, S. M.; Binkoski, A. E.; Hilpert, K. F.; Griel, A. E.; Etherton, T. D., Bioactive compounds in foods: Their role in the prevention of cardiovascular disease and cancer. *Am. J. Med.* **2002,** *113*, 71-88.

[24] Cadahia, E.; Conde, E.; de Simon, B. F.; Garcia-Vallejo, M. C., Changes in tannic composition of reproduction cork *Quercus suber* throughout industrial processing. *J. Agric. Food Chem.* **1998,** *46*, 2332-2336.

[25] Scalbert, A., Antimicrobial properties of tannins. *Phytochemistry* **1991,** *30*, 3875-3883.

[26] Pizzi, A., Tannin-based adhesives. *J. Macromol. Sci. R. M. C.* **1980,** *C18*, 247-315.

[27] Sousa, A. F.; Pinto, P.; Silvestre, A. J. D.; Neto, C. P., Triterpenic and other lipophilic components from industrial cork byproducts. *J. Agric. Food Chem.* **2006,** *54*, 6888-6893.

[28] Santos, S. A. O.; Freire, C. S. R.; Domingues, M. R. M.; Silvestre, A. J. D.; Neto, C. P., Characterization of phenolic components in polar extracts of *Eucalyptus globulus* Labill. bark by high-performance liquid chromatography-mass spectrometry. *J. Agric. Food Chem.* **2011,** *59*, 9386-9393.

[29] Santos, S. A. O.; Villaverde, J. J.; Freire, C. S. R.; Domingues, M. R. M.; Neto, C. P.; Silvestre, A. J. D., Phenolic composition and antioxidant activity of *Eucalyptus grandis*, *E. urograndis* (*E. grandis* x *E. urophylla*) and *E. maidenii* bark extracts. *Ind. Crop. Prod.* **2012,** *39*, 120-127.

[30] Singleton, V. L.; Rossi, J. A., Jr., Colorimetry of Total Phenolics with Phosphomolybdic-Phosphotungstic Acid Reagents. *Am. J. Enol. Vitic.* **1965,** *16*, 144-158.

[31] Sharma, O. P.; Bhat, T. K., DPPH antioxidant assay revisited. *Food Chem.* **2009,** *113*, 1202-1205.

[32] Velioglu, Y. S.; Mazza, G.; Gao, L.; Oomah, B. D., Antioxidant Activity and Total Phenolics in Selected Fruits, Vegetables, and Grain Products. *J. Agric. Food Chem.* **1998,** *46*, 4113-4117.

[33] He, Z.; Xia, W., Analysis of phenolic compounds in Chinese olive (*Canarium album* L.) fruit by RPHPLC–DAD–ESI–MS. *Food Chem.* **2007,** *105*, 1307-1311.

[34] Falcao, S. I.; Vilas-Boas, M.; Estevinho, L. M.; Barros, C.; Domingues, M. R. M.; Cardoso, S. M., Phenolic characterization of Northeast Portuguese propolis: usual and unusual compounds. *Anal. Bioanal. Chem.* **2010,** *396*, 887-897.

[35] Sandhu, A. K.; Gu, L. W., Antioxidant Capacity, Phenolic Content, and Profiling of Phenolic Compounds in the Seeds, Skin, and Pulp of *Vitis rotundifolia* (Muscadine Grapes) As Determined by HPLC-DAD-ESI-MS^n. *J. Agric. Food Chem.* **2010,** *58*, 4681-4692.

[36] Fabre, N.; Rustan, I.; de Hoffmann, E.; Quetin-Leclercq, J., Determination of flavone, flavonol, and flavanone aglycones by negative ion liquid chromatography electrospray ion trap mass spectrometry. *J. Am. Soc. Mass Spectr.* **2001,** *12*, 707-715.

[37] Fernandes, A.; Fernandes, I.; Cruz, L.; Mateus, N.; Cabral, M.; de Freitas, V., Antioxidant and biological properties of bioactive phenolic compounds from *Quercus suber* L. *J. Agric. Food Chem.* **2009,** *57*, 11154-11160.

Chapter 6

General discussion and concluding remarks

6.1 General discussion

In the Introduction of this thesis (Chapter 1), the importance of research, in order to upgrade the vast range of agro-forest by-products generated, not only in Portugal, but also elsewhere, was highlighted. It was also emphasised the importance of studying the chemical composition of those by-products, in order to exploit them as sources of valuable compounds, in particular of phenolic compounds (Chapter 2).

In the first part of the experimental work developed in this thesis (Chapter 3), the phenolic fraction of *Eucalyptus globulus* bark was analysed. Conventional solid-liquid extraction was applied (Part A), considering published data [1-3]. Therefore, two solvents/solvent mixtures were used in the extraction: a mixture of methanol:water (50:50), and methanol, followed by water. The extracts obtained were analysed by their extraction yield, total phenolic content by Folin-Ciocalteu method and by identification and quantification of their phenolic components. The separation of the compounds was achieved by HPLC and the identification was done by two types of mass spectrometry techniques: tandem mass spectrometry, obtained in a triple-quadrupole, and multi-stage mass spectrometry, using an ion trap spectrometer.

The extraction yield of *E. globulus* bark obtained with the single step MeOH/H_2O accounted 9.28%, a value lower than the sum of the extraction yields for MeOH (8.24%) and water (1.93%). The analysis of the TPC of each extract allowed to verify that MeOH/H_2O and MeOH extracts have a similar TPCs (413.8±5.27 and 409.7±2.76 mg GAE g^{-1}, respectively), while the water extract showed a significantly lower amount (115.3±0.50 mg GAE g^{-1}). However, considering the values in a basis of mass of bark, a TPC value of about 38.4 g GAE Kg^{-1} of bark can be achieved with a MeOH/H_2O extraction, a value higher than the sum of TPCs with sequential extraction with MeOH (~33.8 g GAE Kg^{-1} of bark) and water (~2.2 g GAE Kg^{-1} of bark).

The analysis of the extracts by HPLC-MS allowed to identify twenty-nine phenolic compounds, 16 of them referenced for the first time as constituents of *E. globulus* bark. Namely, quinic, dihydroxyphenylacetic, and caffeic acids, bis-HHDP-glucose, galloyl-bis-HHDP-glucose, galloyl-HHDP-glucose, isorhamnetin-hexoside, quercetin-hexoside, methyl-ellagic acid -pentoside, myricetin-rhamnoside, isorhamnetin-rhamnoside, mearnsetin, phloridzin, mearnsetin-hexoside, luteolin and a proanthocyanidin B-type dimer. From a qualitative point of view, the same phenolic compounds were identified in both MeOH/H_2O and MeOH extracts. The single MeOH/H_2O extraction step shows abundances of the identified compounds in the extracts globally higher than those

obtained with MeOH, but lower than the total obtained in the sequential extraction with MeOH followed by water. However, as was verified by TPC, the total amount of identified compounds per mass of bark is clearly higher with a MeOH/H_2O extraction (~10.9 g kg^{-1}) than the sum of the other two (~8.4 g kg^{-1}), clearly demonstrating the advantage of this single step extraction. Digalloylglucose is the main compound in the MeOH (17.95 mg g^{-1} of extract) and MeOH/H_2O (17.77 mg g^{-1} of extract) extracts, followed by isorhamnetin-rhamnoside (9.79 mg g^{-1} of extract) and galloyl-HHDP-glucose (9.27 mg g^{-1} of extract) in the MeOH extract and by catechin (14.23 mg g^{-1} of extract) and chlorogenic acid (13.36 mg g^{-1} of extract) in the MeOH/H_2O extract. The water extract was found to have considerably lower amounts of phenolic compounds, with catechin (15.94 mg g^{-1} of extract), galloyl-HHDP-glucose (9.04 mg g^{-1} of extract) and digalloylglucose (6.35 mg g^{-1} of extract) as the major components. Considering the phenolic content values in a bark basis, digalloylglucose (~1.65 g Kg^{-1} of bark), catechin (~1.32 g Kg^{-1} of bark) and chlorogenic acid (1.24 g Kg^{-1} of bark) are the major phenolic compounds present in *E. globulus* bark (Figure 6.1).

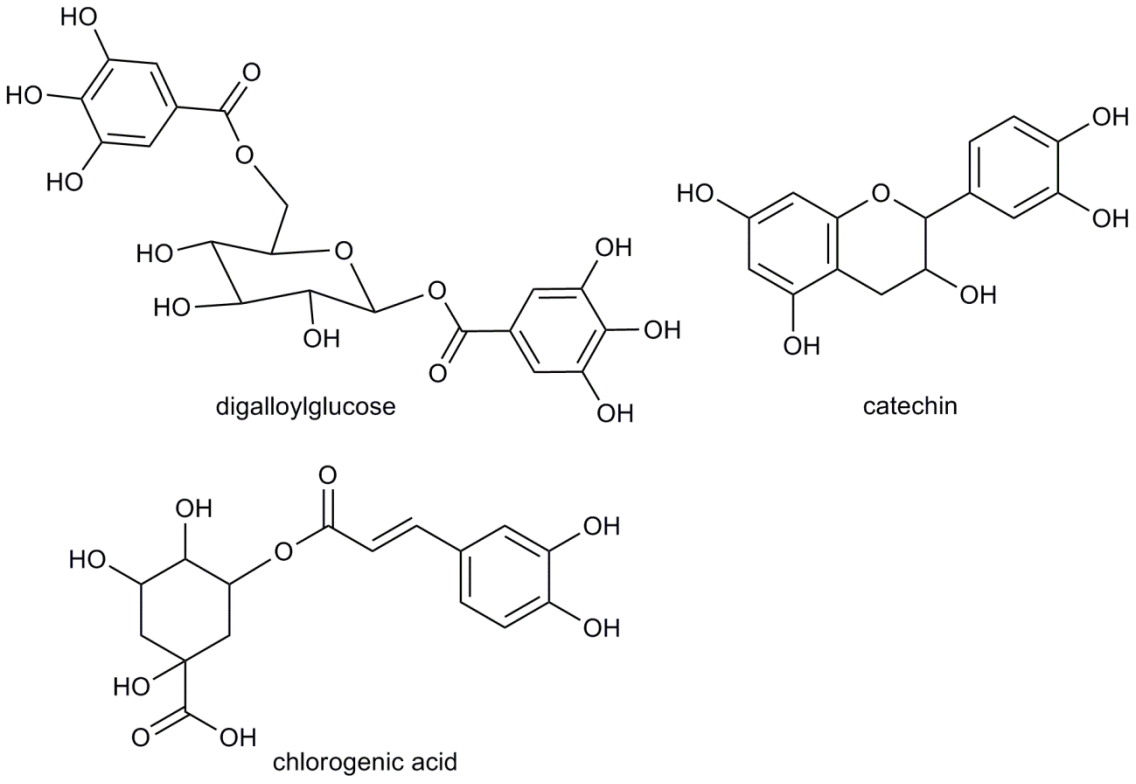


Figure 6.1 – Major phenolic compounds identified in *E. globulus* bark

This study was successful for several reasons. On the one hand, the number of phenolic compounds detected and identified with HPLC and MS. On the other hand, it was possible to verify that a single step extraction is efficient in the extraction of

phenolic compounds from *E. globulus* bark. Last, but not the least, the number of new phenolic compounds identified for the first time as constituents of *E. globulus* bark is also particularly relevant.

In the second part of Chapter 3 (Part B) the conditions for the SFE-CO_2 extraction were optimised. This study started with the analysis of the effect of the co-solvent in the extracts. Therefore experiments without co-solvent and by using water, ethyl acetate and ethanol. This study was complemented with conventional soli-liquid extractions with methanol/water and ethanol/water mixtures, for comparative purposes, since the bark samples used in this part were from a distinct sampling period of those used in Part A. However, the extraction yield of MeOH/H_2O SLE extract were comparable with those obtained in Part A (9.28%). In the same way similar values were detected in TPC (407.41±16.68 against 413.8±5.27 mg GAE g^{-1} of extract).

Concerning the conventional SLE extraction with EtOH/H_2O, an extraction yield slightly higher (9.74%) than with MeOH/H_2O was obtained, although with a lower amount of TPC (159.57±6.75 mg GAE g^{-1} of extract). The extracts were also analysed for their antioxidant activity by DPPH radical assay. Both MeOH/H_2O and EtOH/H_2O extracts showed IC_{50} values between those determined by BHT and ascorbic acid. Once more, MeOH/H_2O extraction shows advantages comparatively to EtOH/H_2O extraction, leading to extracts with higher antioxidant activity. When supercritical extraction was used, the extraction yields decreased drastically, down to 0.05%. However, using ethanol as co-solvent shows to be slightly more efficient, since an extraction yield of 0.32% was achieved, against values of 0.05, 0.04 and 0.08% without co-solvent or with water or EtOAc as co-solvents, respectively. The same conclusion can be taken comparing the values of TPC, which range between 9.22±0.27 (CO_2/H_2O) and 33.10±0.53 (CO_2/EtOH) mg GAE g^{-1} of extract.

Regarding the antioxidant activities of the extracts, the CO_2, CO_2/EtOAc and CO_2/H_2O show to have an extremely low antioxidant activity (IC_{50} > 350 µg mL^{-1}), while CO_2/EtOH extract has a higher value (IC_{50}=64.81 µg mL^{-1}), although significantly lower than those obtained with conventional SLE extractions. To finalise this study, CO_2 and CO_2/EtOH extracts were analysed by HPLC-MS. Undoubtedly, ethanol incremented not only quantitatively, but also qualitatively the phenolic compounds extracted. In CO_2 extract only two phenolic compounds were detected (digalloylglucose and naringenin), while in the CO_2/EtOH extract sixteen phenolic compounds were detected and quantified. Surprisingly, this extraction showed to be selective to the flavanones eriodictyol and naringenin and to the *O*-methylated flavonol isorhamnetin (Figure 6.2),

with contents (in mg g^{-1} of extract) higher than that previously achieved with MeOH/H_2O SLE procedure (Chapter 3 –Part A).

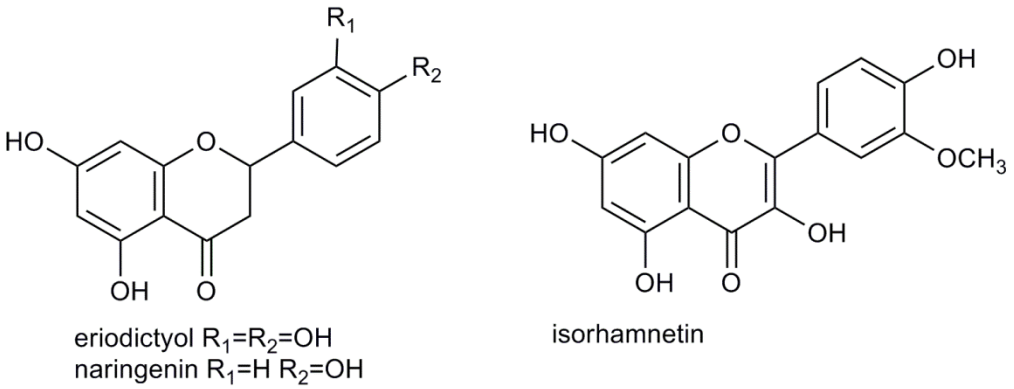


Figure 6.2 – Major phenolic compounds identified in CO_2/EtOH extract of *E. globulus* bark

The CO_2/EtOH extract also presents methyl-ellagic acid, a compound that was not detected in SLE extracts, and it was referenced only as a constituent of *E. globulus* fruits [4]. Therefore, the number of phenolic compounds identified in *E. globulus* bark rises to thirty, with seventeen referenced for the first time as its components.

After the conclusion that ethanol is the best co-solvent for the SFE-CO_2 extraction of the phenolic fraction from *E. globulus* bark, the next step involved the optimisation of the SFE conditions. Therefore, three of the most important parameters in SFE were optimised: temperature, ethanol content and CO_2 flow rate. The effect of these parameters in the extraction yield, total phenolic content, phenolic content quantified by HPLC-UV and antioxidant activity was verified, applying a full 2^3 design of experiments. The ethanol content showed a significant and positive influence in the four responses, while the temperature affected all the responses, with exception of the phenolic content quantified by HPLC. Conversely, the CO_2 flow rate only had influence on total phenolic content. This influence can indicate some problems related on external mass transfer phenomena. This study ended with the determination of the optimal SFE conditions, maximizing all responses: 70 ºC, 20% (wt) of ethanol, and 10 g of CO_2 min^{-1} at 300 bar. The values of the dependent variables at this point were: EY = 0.48% of extraction yield, TPC = 57.22 mg GAE g^{-1} of extract, PC-HPLC = 119.46 mg g^{-1} of extract, and AA = 49.74 mg AAE g^{-1} of extract. Although the extraction yield and antioxidant activity are quite distant from those obtained with conventional SLE, the amount of phenolic compounds quantified by HPLC in mg g^{-1} of extract clearly exceeds that obtained with MeOH:H_2O SLE. It has to be highlighted that this is only in a quantitative point of view,

since the range of compounds detected in SFE and SLE were not the same. Nevertheless, the selectivity of SFE could be also an exploitable task.

In order to verify the potential of other pulp and paper mill by-products generated worldwide as sources of phenolic compounds, the barks of *E. grandis*, *E. urograndis* and *E. maidenii* were analysed (Chapter 4). The conditions of conventional SLE applied in *E. globulus* bark were used. The extraction yields of the barks of the three species varied between 10.54% (*E. grandis*) and 15.18% (*E. urograndis*), which are clearly higher than that reported for *E. globulus*. The TPC values obtained, 385.63±11.02, 346.72±7.76 and 203.86±4.37 mg GAE g^{-1} of extract, for *E. grandis*, *E. urograndis* and *E. maidenii*, respectively, are in the same range, although lower than that obtained for *E. globulus* bark. The analysis by HPLC-MS allowed to verify that some compounds are constituents of all the species analysed, including *E. globulus*, namely quinic, gallic, protocatechuic and ellagic acids and methyl gallate, catechin, galloyl-bis-HHDP-glucose and isorhamnetin-rhamnoside. Furthermore, three new phenolic compounds were identified as constituents of *Eucalyptus* spp. bark, namely ellagic acid-rhamnoside, dihydroxy-isopropylchromone-hexoside and dihydroxy-(methylpropyl)isopropylchromone-hexoside.

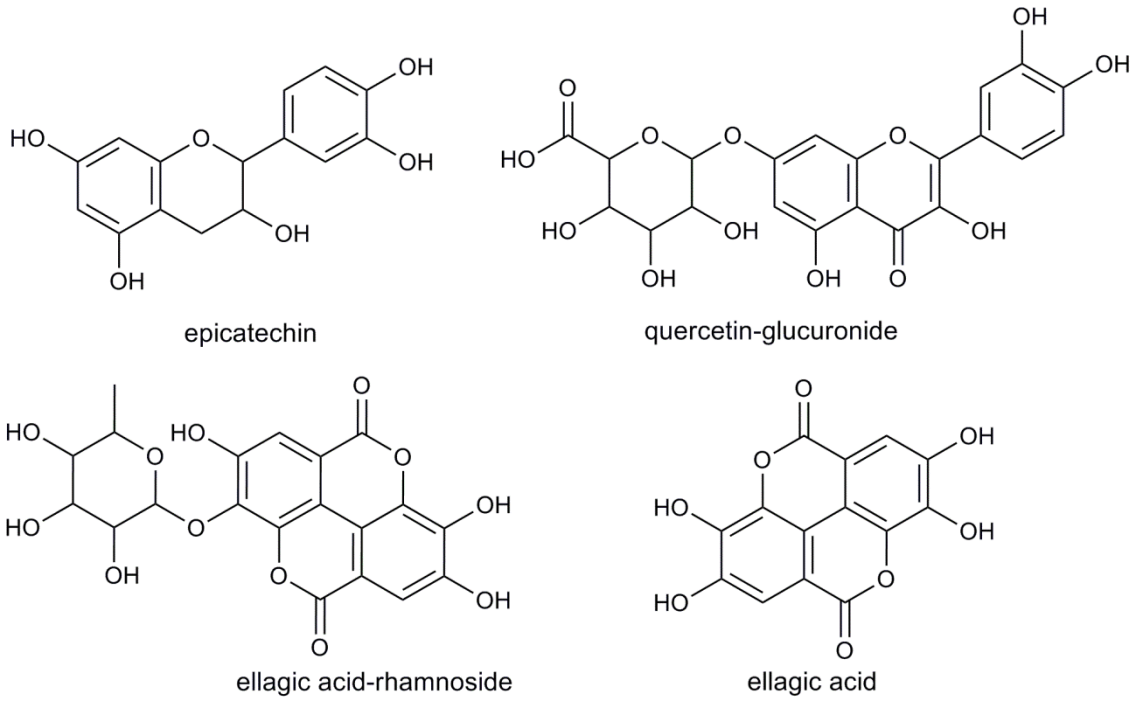


Figure 6.3 – Major phenolic compounds identified in *E. grandis* bark

Epicatechin and quercetin-glucuronide (quantified together) are the main constituents of the phenolic fraction of *E. grandis* and *E. urograndis*, followed by ellagic acid-

rhamnoside and ellagic acid in *E. grandis* (Figure 6.3) and by galloyl-bis-HHDP-glucose and gallic acid in *E. urograndis* (Figure 6.4).

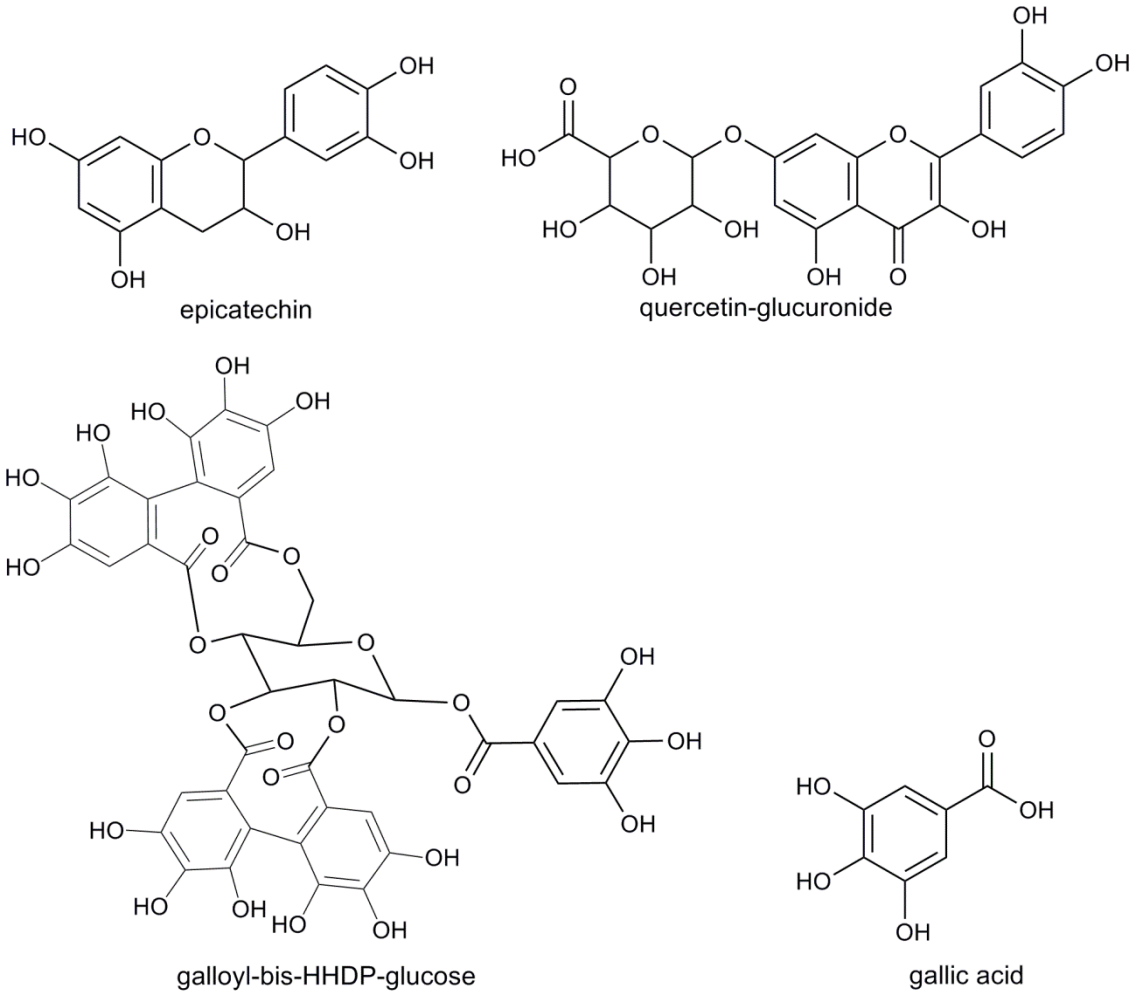


Figure 6.4 – Major phenolic compounds identified in *E. urograndis* bark

Considering the total amount of phenolic compounds quantified by HPLC, those two species showed similar values and higher than those of *E. maidenii*. Nevertheless, *E. maidenii* showed a larger diversity on the phenolic composition, with a total of twenty four phenolic compounds being detected, against thirteen and twelve detected in *E. grandis* and *E. urograndis*, respectively. In addition, the main compounds quantified were catechin, chlorogenic acid and methyl-ellagic acid-pentoside (Figure 6.5).

Concerning the antioxidant activity of the extracts, the values determined are all in the same range, varying the IC_{50} values between 6.14±0.21 and 8.24±0.26 μg mL^{-1}. The AA of these three extracts are clearly lower than that of *E. globulus* bark extract, although the IC_{50} values are still between those of the two commercial antioxidants BHT and ascorbic acid. This study was pioneer, regarding the phenolic fraction of the

bark from these species, and, clearly, shows the potential of these by-products as sources of valuable phenolic compounds.

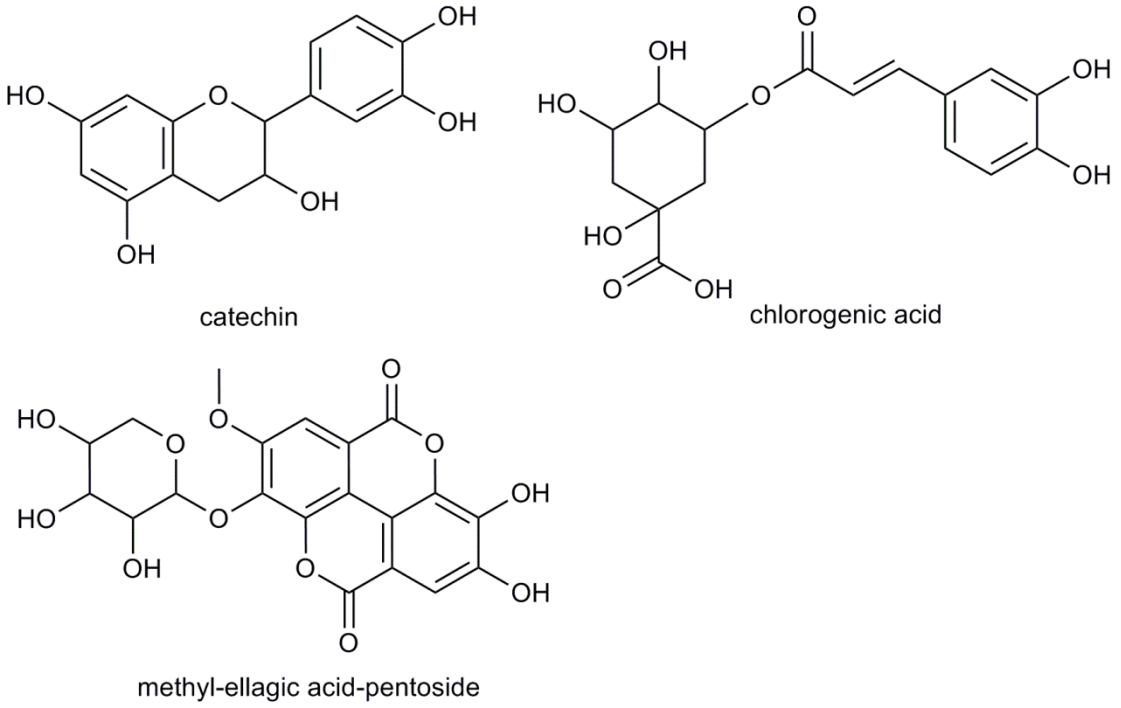


Figure 6.5 – Major phenolic compounds identified in *E. maidenii* bark

The Chapter 5 deals with the analysis of phenolic fraction of cork and related by-products, and was divided in two parts. In the first part (Chapter 5-Part A), only the *Quercus suber* cork was analysed, in order to study in detail its phenolic composition. Conventional SLE with methanol:water (80:20), followed by a liquid-liquid extraction with diethyl ether were applied [5, 6]. In addition, a sequential extraction, first with methanol and then with water, was also performed.

The extraction yields obtained for methanol/water (80:20) and methanol extracts were similar (1.2 and 1.7%), while the water extract showed a higher yield (3.7%). The analysis of the three extracts by HPLC-MS allowed to identify fifteen phenolic compounds, between phenolic acids, a coumarin and flavonoids, in particular, flavanones. Five of the compounds identified were reported for the first time as cork constituents, namely, quinic and salicylic acids, *p*-hydroxyphenyllactic acid, eriodictyol and naringenin. All the extracts have ellagic acid (Figure 6.6) as the main constituent, with contents of 2031.5 mg Kg^{-1} of dry cork obtained with MeOH/H_2O (80:20) extraction and 2103.4 mg Kg^{-1} obtained with MeOH extraction followed by water. Furthermore, cork showed also high contents of gallic acid (289.7 mg Kg^{-1} of dry cork, obtained with

MeOH extraction followed by water) and protocatechuic acid (177.3 mg Kg^{-1} of dry cork, obtained with MeOH extraction, followed by water).

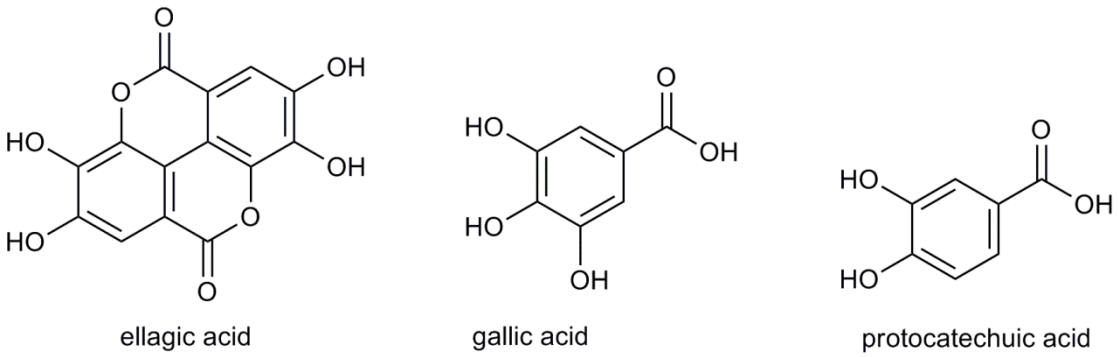


Figure 6.6 – Major phenolic compounds identified in MeOH/H_2O (80:20), MeOH and water extracts of *Q. suber* cork

The cork extracts were also analysed by their antioxidant activity, using ascorbic acid and BHT as reference compounds. Diethyl ether extract showed the highest AA (even higher than ascorbic acid). The methanol and water extracts also showed promising values of antioxidant activity, with IC_{50} values between those of ascorbic acid and BHT. Due to its high extraction yield, the AA of water extract become higher than the other extracts, when expressed as ascorbic acid equivalents in a dry cork basis. This result could be interesting to exploit water extracts of cork, in a perspective of a source of valuable compounds.

The second part of this Chapter focused on the analysis of phenolic fraction from industrial cork by-products (Chapter 5-Part B). However, taking into account the successful MeOH/H_2O SLE obtained previously for *E. globulus* bark, and then for the other three *Eucalyptus* spp., the same methodology was applied in cork and related by-products. In addition, these extracts were analysed by multi-stage mass spectrometry. The extraction yield of cork is clearly higher (5.93%) than those obtained previously with other SLE methodologies. The same behaviour was achieved for TPC, despite being in the same range. The EY of ICP and BC were slight lower than for NC, accounting values of 2.26 and 0.99%, respectively. These extracts show TPC values of 254.54±0.28 and 167.32±0.61 mg g^{-1} of extract, which are lower than that achieved for cork.

In this study, a total of eighteen different phenolic compounds were detected in cork, five of them refereed for the first time as cork constituents. Namely, methyl gallate, brevifolin carboxylic acid, caffeic acid isoprenyl ester, isorhamnetin-rhamnoside and isorhamnetin. The identification of most of the compounds in this study was possibly

due to the use of multi-stage MS. In fact, Fernandes and co-workers [7] also identified several more complex phenolic compounds, some of them also identified in this study, by using the same mass spectrometry technique.

It has to be highlighted that the phenolic content quantified by HPLC of *Q. suber* cork obtained with this extraction procedure, in mg g^{-1} of extract, is lower than that obtained with MeOH/H_2O (80:20) extraction, followed by diethyl ether liquid/liquid extraction (Chapter 5-Part A). However, when it is considered the amount of phenolic compounds in a dry cork basis, the MeOH/H_2O (50:50) extraction allowed to obtain clearly higher values (~4.25 against 2.22 g Kg^{-1} of dry cork). Moreover, and apart from the new phenolic compounds identified, this extraction allowed to obtain higher amounts of each compound, with the exception of ellagic acid, than those obtained with MeOH/H_2O (80:20) extraction. Notwithstanding, ellagic acid was identified as the major component of phenolic fraction of *Q. suber* cork, followed by ellagic-acid pentoside and ellagic acid-rhamnoside (quantified together) and gallic acid (Figure 6.7). The content of ellagic acid derivatives, together with the amount of free ellagic acid, could be interesting and an exploitable task in cork valorisation.

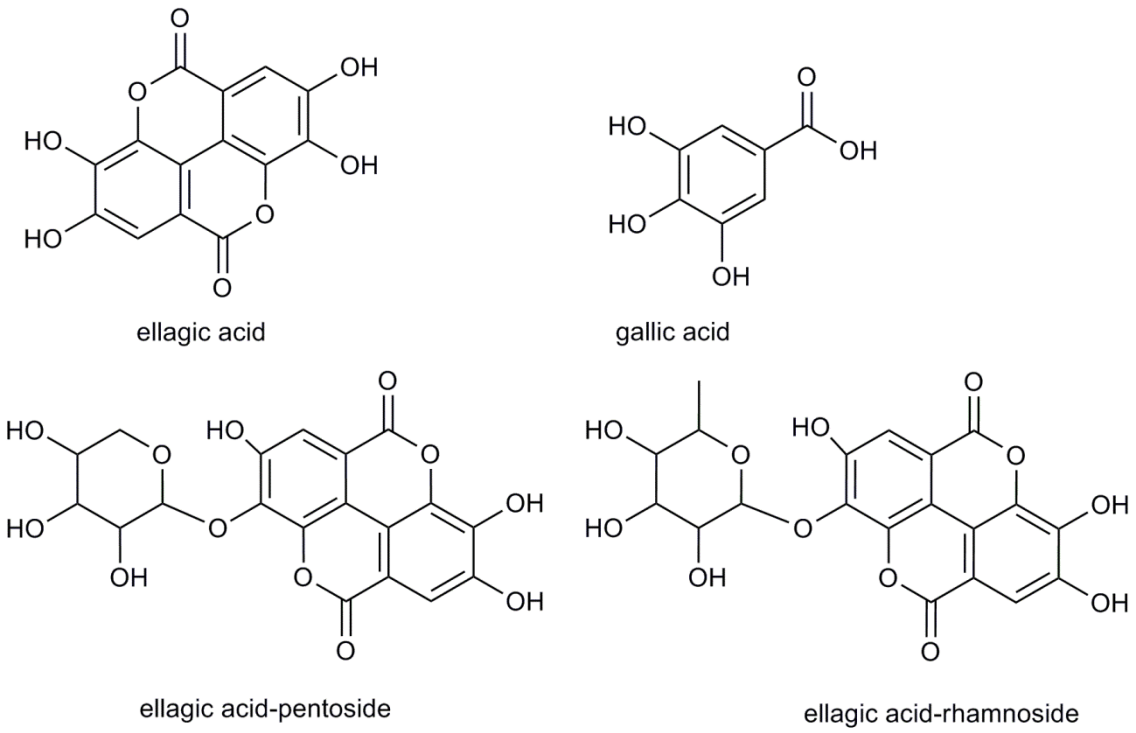


Figure 6.7 – Major phenolic compounds identified in MeOH/H_2O (50:50) extract of *Q. suber* cork

Sixteen phenolic compounds were identified in ICP, namely, quinic, gallic, protocatechuic, caffeic, ferulic and ellagic acids and methyl gallate, esculetin, brevifolin carboxylic acid, coniferaldehyde, caffeic acid isoprenyl ester, valoneic acid dilactone, ellagic acid-pentoside, ellagic acid-rhamnoside, isorhamnetin-rhamnoside and isorhamnetin. Among these, only ellagic acid was previously reported as constituent of ICP. In the same way, thirteen phenolic compounds were identified in black condensate, twelve of them for the first time. Namely, quinic, gallic, *p*-hydroxypheyllactic, protocatechuic, *p*-coumaric, caffeic and ellagic acids and vanillin, esculetin, coniferaldehyde, caffeic acid isoprenyl ester and eriodictyol. Cork powder has also ellagic acid as the majority compound, followed by gallic acid and esculetin (Figure 6.8).

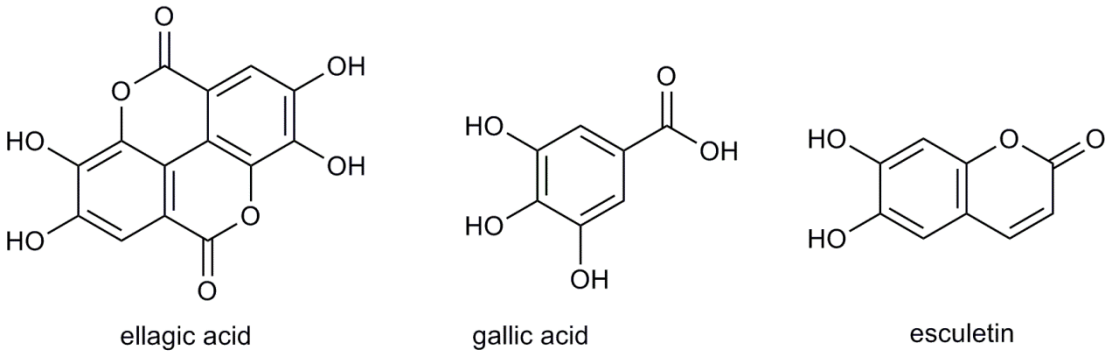


Figure 6.8 – Major phenolic compounds identified in industrial cork powder

Coniferaldehyde, esculetin and gallic acid (Figure 6.9) are the major compounds of black condensate, showing this extract a total amount of phenolic compounds quantified by HPLC, when expressed as mg g^{-1} of extract, in the same range of those obtained for ICP and NC. This is a promising result, since it shows that this unexploited by-product could be a valuable source of phenolic compounds.

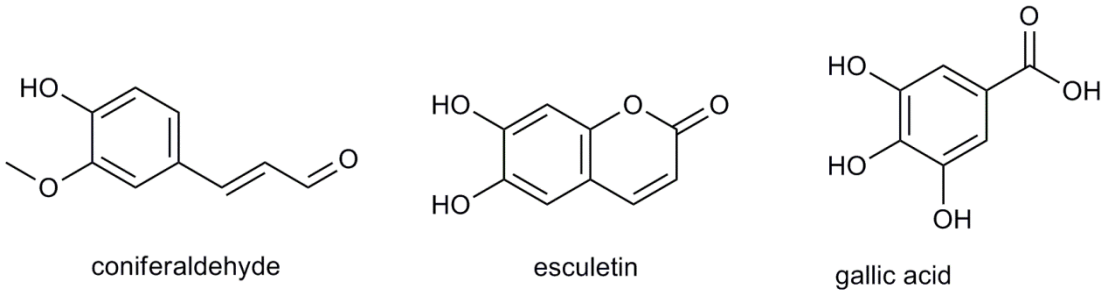


Figure 6.9 – Major phenolic compounds identified in black condensate

Furthermore, promising results were obtained analysing their antioxidant activity, which is higher than that for BHT and in the range of than that for ascorbic acid. Interestingly,

black condensate has a higher AA than ascorbic acid, and, inclusively, the highest AA achieved in all the biomass fractions analysed in this work. The antioxidant activities of ICP also showed promising results, with an IC_{50} value (3.33±0.02 μg mL^{-1}), lower than that for NC (4.77±0.02 μg mL^{-1}). The AA of cork is in line with those obtained from the others biomass analysed. In addition, both IC_{50} values for NC and ICP are between those of ascorbic acid and BHT.

6.2 Concluding remarks and future work

This study allowed to verify that several agro-forest industrial by-products can, clearly, be exploited as sources of valuable phenolic compounds.

One of the main conclusions to be highlighted is the vast range of phenolic compounds, from different classes, identified, by combining the advantages of high-performance liquid chromatography and distinct mass spectrometry techniques.

Eucalyptus globulus, *E. grandis*, *E. urograndis* and *E. maidenii* barks show to be constituted by a vast range of phenolic compounds, including phenolic acids and aldehydes, chromones, different groups of flavonoids, flavonoids glycosides, ellagic acid and galloylglucose derivatives and inclusively ellagitannins.

In the same way, the industrial cork by-products, namely, industrial cork powder and black condensate, present a wide variety of interesting phenolic compounds, involving phenolic acids and aldehydes, cinnamic acids, coumarins, ellagic acid derivatives and flavonoids.

Cork could also be up-graded and exploited as a source of valuable phenolic compounds and, particularly, as a source of ellagic acid.

All the extracts analysed showed promising antioxidant activities, which, in most of the cases, could even compete with the antioxidant activities of commercially available antioxidants, such as BHT and ascorbic acid. Black condensate, in particular, shows the lowest IC_{50} value of all the biomasses analysed, and even lower than BHT and ascorbic acid.

Supercritical fluid extraction of phenolic compounds from *E. globulus* bark, although leading to low extraction yields, showed to be selective for restricted classes of phenolic compounds, namely, flavanones (eriodictyol and naringenin) and one *O*-methylated flavonol (isorhamnetin). This can be further exploited for specific uses, where these families of compounds might be targeted.

This study constitutes an important step in the up-grading of these industrial residues, however, several further steps need to be developed before industrial/commercial exploitation becomes real.

In the case of *E. globulus* bark, supercritical fluid extraction should be applied after an environmentally friendly extraction of lipophilic components, instead of dichloromethane extraction. In this vein, recently Domingues and co-workers [8] have applied and optimised SFE extraction of lipophilic components from *E. globulus* bark. In the same way, supercritical fluid extraction should also be applied to the pre-extraction of the other by-products and the conditions also optimised.

The extracts obtained could also be evaluated concerning other activities, beside antioxidant activity. In fact, Mota *et al.* [9] showed, recently, the promising potentialities of *E. globulus* bark extracts as anti-proliferative agents in human breast cancer cell lines.

At last, but probably one of the most important tasks to be taken in a future work, includes the development of methodologies to purify the fractions/compounds of interest.

References

[1] Vázquez, G.; Fontenla, E.; Santos, J.; Freire, M. S.; González-Álvarez, J.; Antorrena, G., Antioxidant activity and phenolic content of chestnut (*Castanea sativa*) shell and eucalyptus (*Eucalyptus globulus*) bark extracts. *Ind. Crop. Prod.* **2008,** *28*, 279-285.

[2] Conde, E.; Cadahia, E.; DiezBarra, R.; GarciaVallejo, M. C., Polyphenolic composition of bark extracts from *Eucalyptus camaldulensis*, *E. globulus* and *E. rudis*. *Holz Als Roh- und Werkst.* **1996,** *54*, 175-181.

[3] Conde, E.; Cadahía, E.; García-Vallejo, M.; Tomás-Barberán, F., Low molecular weight polyphenols in wood and bark of *Eucalyptus globulus Wood Fiber Sci.* **1995,** *27*, 379-383.

[4] Boulekbache-Makhlouf, L.; Meudec, E.; Chibane, M.; Mazauric, J. P.; Slimani, S.; Henry, M.; Cheynier, V.; Madani, K., Analysis by High-Performance Liquid Chromatography Diode Array Detection Mass Spectrometry of Phenolic Compounds in Fruit of Eucalyptus globulus Cultivated in Algeria. *J. Agric. Food Chem.* **2010,** *58*, 12615-12624.

[5] Conde, E.; Cadahía, E.; García-Vallejo, M. C.; de Simón, B. F.; Adrados, J. R. G., Low molecular weight polyphenols in cork of *Quercus suber*. *J. Agric. Food Chem.* **1997,** *45*, 2695-2700.

[6] Conde, E.; Cadahia, E.; Garcia-Vallejo, M. C.; de Simon, B. F., Polyphenolic composition of *Quercus suber* cork from different Spanish provenances. *J. Agric. Food Chem.* **1998,** *46*, 3166-3171.

[7] Fernandes, A.; Sousa, A.; Mateus, N.; Cabral, M.; de Freitas, V., Analysis of phenolic compounds in cork from *Quercus suber* L. by HPLC–DAD/ESI–MS. *Food Chem.* **2011,** *125*, 1398-1405.

[8] Domingues, R. M. A.; Oliveira, E. L. G.; Freire, C. S. R.; Couto, R. M.; Simoes, P. C.; Neto, C. P.; Silvestre, A. J. D.; Silva, C. M., Supercritical Fluid Extraction of Eucalyptus globulus Bark-A Promising Approach for Triterpenoid Production. *Int. J. Mol. Sci.* **2012,** *13*, 7648-7662.

[9] Mota, I.; Rodrigues Pinto, P. C.; Novo, C.; Sousa, G.; Guerreiro, O.; Guerra, A. R.; Duarte, M. F.; Rodrigues, A. E., Extraction of polyphenolic compounds from *Eucalyptus globulus* cark: Process optimization and screening for biological activity. *Ind. Eng.Chem. Res.* **2012,** *51*, 6991-7000.